The Cochaperone and Ubiquitin ligase CHIP in Protein Quality Control

Qian Dai

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology.

Chapel Hill
2006

Approved by
Advisor: Cam Patterson
Reader: Channing Der
Reader: David Siderovski
Reader: Doug Cyr
Reader: Yue Xiong

ABSTRACT

QIAN DAI: The Cochaperone and Ubiquitin ligase CHIP in Protein Quality Control

(Under the direction of Cam Patterson)

Protein quality control is essential for living cells to maintain homeostasis during normal growth conditions as well as upon stress challenges. Molecular chaperones and the ubiquitin-proteasome system are the two arms of the cellular protein quality control system. CHIP is an Hsp70/Hsp90 cochaperone that inhibits ATPase activity of chaperones and enhances protein folding in vivo. CHIP is also a chaperone-dependent E3 ubiquitin ligase that diverts chaperone substrates to the proteasome. Therefore, CHIP regulates both arms of the protein quality control system.

My studies have established that CHIP regulates the stress-chaperone response through induced trimerization and transcriptional activation of HSF1, which leads to transcriptional upregulation of heat shock proteins. This upregulation is required for cells to cope with stress as *CHIP* (–/–) murine fibroblasts have decreased viability and increased apoptosis after heat shock or protein damaging agent treatment. Activation of HSF1 by CHIP is essential for the cells to cope with stress challenges.

E3 ubiquitin ligase activity of CHIP has been implicated in the degradation of a variety of chaperone-bound cytoplasmic proteins. Using a proteomics approach, we have identified BAG2 as a common component of CHIP holocomplexes in vivo. Binding assays indicate that BAG2 associates with CHIP as part of a ternary complex with Hsc70.

BAG2 is an efficient and specific inhibitor of CHIP-dependent ubiquitin ligase activity. This activity is due, in part, to inhibition of interactions between CHIP and its cognate ubiquitin-conjugating enzyme, UbcH5a. The association of BAG2 with CHIP provides a cochaperone-dependent regulatory mechanism for preventing unregulated ubiquitylation of misfolded proteins by CHIP.

Taken together, my work has established the mechanism of CHIP to regulate the stress response and identified a regulatory factor for its E3 ubiquitin ligase activity.

In memory of my mother, Qingzhu Chen

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Cam Patterson, for giving me the opportunity to complete my graduate study in his lab. I was originally recruited by the University of Texas Medical Branch at Galveston and was doing my lab rotation in the Patterson lab there, when the lab moved to UNC Chapel Hill. It was with the generous help of Dr. Patterson and the Pharmacology department of UNC Chapel Hill, that I could transfer to UNC Chapel Hill and finish my graduate study. Dr. Patterson's devotion and passion for research will have a significant impact to my career in the future.

I would like to thank Dr. Madamanchi Nageswara of the Runge lab. Dr. Nageswara taught me basic skills of scientific research when I first came to the Patterson lab and continued to give me intellectual and technical support throughout my graduate study. I would also like to thank: Dr. Jihong Jiang, a former graduate student in the Patterson lab, for ideas and discussions on our research topic; Dr. Chunlian Zhang and Ryan Whaley, for their assistance with animal experiments; Drs. Jae Hwang, Shu-Bing Qian, Hui-hua Li, Ms Yaxu Wu and Holly McDonough, for their generous help with my experiments. Without them, I could not have published two papers during my graduate study; and everyone in the Patterson lab, for creating an enjoyable research environment that I benefited from; Ms. Hong-Yu Ren of Dr. Doug Cyr lab, for her generous help with protein purification and ubiquitination assays.

I would like to thank my dissertation committee, Dr. Channing Der, Dr David Siderovski, Dr. Doug Cyr and Dr. Yue Xiong, for their questions and suggestions to my

research. I would especially thank Dr. Cyr for his intellectual input into and generous provision of experimental reagents for my second project, the CHIP-BAG2 interaction story.

I would like to thank my family members: my parents, Guoliang Dai and Qingzhu Chen, who instilled in me my love for learning; my husband, Dr. Hengbin Wang, who gives me continuous support, invaluable discussions and endless encouragements for my research; my three-year-old son, David Wang, who has been a wonderful kid and generated endless happiness for our family; my brother, Dr. Hao Chen, who gave me numerous suggestions on my personal and academic life when I first came to the United States by myself.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

17-AAG	17-(Allylamino)-17-desmethoxygeldanamycin
A	alanine
AD	Alzheimer's disease
ADP	adenine diphosphate
ALS	amyotrophic lateral sclerosis
APC	Anaphase Promoting Complex
ATP	adenine triphosphate
ASK1	apoptosis signal-regulating kinase 1
BAG	bcl2-associated athanogene
CDK	cyclin-dependent kinase
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFTRΔF508	CFTR deletion phenylalanine 508
CHIP	carboxyl-terminus of Hsc70-interacting protein
D	aspartate acid
E6-AP	E6-associated protein
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERα	estrogen receptor α
FKBP	FK506 binding protein
G	glycine
GA	geldanamycin

GR	glucocorticoid receptor
GST	glutathione S-transferase
HECT	homologous to E6-AP carboxyl terminus
Hip	Hsc70 interacting protein
Hop	Hsp organizer protein
HPV	human papilloma virus
HS	heat shock
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
HspBP1	heat shock protein binding protein 1
Hsc70	heat shock cognate protein
IP	immunoprecipitation
JNK	c-Jun N-terminal kinase
k48	lysine 48
kDa	kilo-Dalton
MAPKKK	mitogen-activated protein kinase kinase kinase
N	asparagine
NBD	nucleotide binding domain
nNOS	neuronal nitric oxide synthase
PAGE	polyacrylamide gel electrophoresis
Pael-R	Pael receptor
PCR	polymerase chain reaction

PD	Parkinson’s disease
polyQ	polyglutamine
PP5	protein phosphatase 5
RalBP1	Ral-binding protein 1
RING	really interesting new gene
RIPA	radio-immunoprecipitation assay
SCF complexes	Skp1-Cullin-F-box protein complexes
SDS	sodium dodecyl sulfate
SUMO	small ubiquitin-like modifier
TPR	tetratricopeptide repeat
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
VCB	von-Hippel-Lindau-Elongins B and C

Chapter 1

General Introduction

1.1 Molecular chaperones and protein quality control

Molecular chaperones are a family of intracellular proteins that are responsible for folding newly synthesized proteins into proper structure and sending damaged proteins for degradation. In 1973 Anfinsen demonstrated the correct (re)folding of RNase upon removal of denaturant, suggesting that all the information needed for proper (re)folding was contained in the primary sequence of the protein(Anfinsen, 1973). However, this conclusion cannot be extrapolated to living cells. The major difference between the in vitro and in vivo system is the highly crowded environment inside the cells due to soluble and insoluble macromolecules, which makes spontaneous folding of proteins inside the cells unfavorable.

Molecular chaperones have evolved to bind exposed hydrophobic patches on proteins, prevent them from aggregating into insoluble, nonfunctional inclusions and help them reach their stable native state. Molecular chaperones comprise several families of heat shock proteins, including the Hsp100, Hsp90, Hsp70, Hsp40, chaperonins, and small Hsps family proteins. Members of the Hsp100 family include the bacterial Clp proteins, the plant Hsp101 and yeast Hsp104. They have been implicated in protein unfolding/disaggregation as well as direct degradation of their substrates by association with proteases (Horwich et al., 1999; Wickner et al., 1999). The chaperonins are a conserved class of large double-ring complexes of ~800 kDa enclosing a central cavity. It contains two groups. Group I chaperonins, also known as GroEL in bacteria or Hsp60 in eukaryotes, are generally found only in eubacteria and in organelles of endosymbiotic origin–mitochondria and chloroplasts. Group II chaperonins exist in the archaeal and the eukaryotic cytosol. The two groups are distinct in primary sequences but are similar in their architecture and function. They capture nonnative proteins through hydrophobic contacts with multiple chaperonin subunits and translocate

them into their central ring cavity where they fold them and protect them from aggregation with other nonnative proteins (Hartl and Hayer-Hartl, 2002). The small Hsp family is also called the HspB family, which contains 10 members from HspB1 to HspB10. This family of Hsps is not associated with ATP and has no protein folding activity. A representative of this family is HspB1/Hsp27. Although it cannot fold denatured proteins, it can adsorb heat-denatured proteins, keep them in a folding-competent state and prevent them from aggregation (Ehrnsperger et al., 1997).

In this introduction, I will focus on Hsp70 and Hsp90 family proteins because of their importance in eukaryotic protein quality control and their physical and functional association with CHIP (Carboxyl terminus of Hsc70 Interaction Protein), the protein our lab has been interested in and has worked on for years.

1.1.1 The Hsp70 family proteins and their cochaperones

The Hsp70 family of chaperones is comprised of bacterial DnaK, yeast Ssa, Ssb, Ssc, mammalian stress-inducible Hsp70 and the constitutive cognate protein Hsc70, mitochondrial mtHsp70/Grp74 and ER Bip/Grp78. This family of proteins functions by binding and release of the extended nonnative polypeptide in an ATP dependent manner, therefore preventing nonnative protein aggregation and promoting their (re)folding. Hsp70 contains a 45 kDa N-terminal ATPase domain, an 18 kDa central substrate binding domain, and a 10 kDa C-terminal domain. The structure and function of the Hsp70 family proteins was first demonstrated in the bacterial DnaK (Hsp70 homolog) and its cochaperone DnaJ (Hsp40 homolog) and GrpE (functional homolog of Hsp40). The ATPase domain of DnaK contains two lobes separated by a cleft where ATP is bound. The substrate binding domain of DnaK is divided into a β-sandwich subdomain with a peptide-binding cleft and an α-helical

latchlike segment (Zhu et al., 1996). DnaK binds to its substrates which have extended polypeptide chains, and forms hydrophobic interactions and hydrogen bonds with the side chain and backbone of its substrates, respectively (Zhu et al., 1996). The affinity of the DnaK substrate binding domain with its substrates is dependent on the ATP/ADP state of its nucleotide binding domain. In the ATP bound state, the α-helical latch is in an open position, allowing substrates to bind to the peptide binding domain. However, in this state, the substrate can also easily dissociate with the substrate binding domain. Therefore, the overall affinity of DnaK with its substrates is low in the ATP binding state. Tight binding of the substrates is triggered by the hydrolysis of ATP into ADP and closure of the α-helical latch, which results in the high-affinity substrate binding state (Bukau and Horwich, 1998; Young et al., 2004). The intrinsic ATPase activity of DnaK is low, and the ATP-ADP cycle is regulated by a group of Hsp70 interacting proteins called Hsp70 cochaperones.

1.1.1a The Hsp40 family cochaperones

The mammalian Hsp40 family proteins include Hdj-1 and Hdj-2, whose progenitor is E coli DnaJ. Both Hdj-1 and Hdj-2 contain the amino terminal signature J-domain (J-domain are composed of approximately 70 amino acids, including an HPD sequence), which plays an important role in Hsp40-Hsp70 interaction (Kelley, 1998) and an adjacent G/F-rich region; however, only Hdj-2 has a cysteine-rich domain. The N-terminal J domains of Hdj-1 and Hdj-2 bind to the carboxyl terminus of Hsp70 and stimulate the ATPase activity of Hsp70 and thus promote the conversion of ATP-bound, low-substrate-affinity Hsp70 to ADP-bound, high-substrate-affinity Hsp70 (Mayer et al., 2000; Pellecchia et al., 2000). The C-terminal domains of Hdj-1 and Hdj-2 function as a chaperone in recognizing hydrophobic peptides and thus recruit Hsp70 to nascent chains (Sha et al., 2000; Rudiger et al., 2001).

Hdj-1 interacts with Hsp70 to fold nascent polypeptides as they emerge from cytosolic ribosomes and helps protect cells from thermal stress. Hdj-2, which possesses an additional CaaX box, a site for prenylation, has been localized to the cytosolic side of the ER membrane (Meacham et al., 1999). Hdj-2 has been implicated in the folding of newly synthesized cystic fibrosis transmembrane conductance regulator (CFTR). Hdj-2 and Hsc70 interacts with early folding intermediates of CFTR on the cytosolic side of the ER membrane. Hdj-2 and Hsc70 function to prevent misfolding or aggregation of a cytosolic domain of CFTR until the protein is further synthesized and can undergo proper intramolecular interactions necessary for its proper folding (Meacham et al., 1999).

1.1.1b The BAG family cochaperones

The genes for BAG (Bcl2-associated athanogene) family proteins are conserved throughout evolution from yeast to mammals (Takayama et al., 1999). Six members of BAG family proteins, from BAG1 to BAG6, have been identified, with BAG1 as the founding member of the family(Takayama and Reed, 2001). All of the BAG family members contain a BAG domain near their carboxyl terminus, except BAG5, which has four putative BAG domains. The BAG domain is a an evolutionarily-conserved domain containing about 50 amino acids, which allows them to interact with the ATPase domain of Hsp70 and regulates their function (Takayama and Reed, 2001).

Recently the structure of the BAG domain-Hsp70 ATPase complex was resolved, which helps us to better understand the regulation of the BAG domain to the ATPase domain of Hsp70. The BAG domain opens the nucleotide-binding cleft upon binding to the ADP-bound state of Hsp70, therefore promoting nucleotide release from Hsp70 (Briknarova et al., 2001; Sondermann et al., 2001). Because of the excess of ATP over ADP and BAG proteins

in the eukaryotic cytosol, ATP will enter the nucleotide-binding pocket and displace bound BAG proteins, resulting in an acceleration of nucleotide exchange. Therefore, the synergistic action of Hsp40 and BAG protein, which stimulates ATPase activity of Hsp70 and releases ADP from Hsp70 respectively, results in the acceleration of the ATPase cycle of Hsp70 and increased substrate binding and refolding activity of Hsp70. E. coli GrpE, although structurally different from BAG domain, stabilizes opening of the nucleotide-binding cleft of the ATPase domain of DnaK similar to the binding of BAG domain to Hsp70 ATPase domain, and thus releases nucleotide and acts as a nucleotide exchange factor. (Brehmer et al., 2001)

In addition to the conserved BAG domain, BAG family proteins also contain a diversity of additional domains, which allows them to interact with specific target proteins or which targets them to specific locations within cells, and perform functions besides nucleotide exchange. These functions include the regulation of signal transducing proteins and transcription factors important for cell stress responses, apoptosis, proliferation, cell migration and hormone action (Takayama and Reed, 2001)

1.1.1c. CHIP

CHIP (carboxyl terminus of Hsc70-interacting protein) was identified during the screening of the TPR (tetratricopeptide repeat)-containing proteins in the human heart cDNA library by the Patterson lab in 1999 (Ballinger et al., 1999). The TPR domain is a protein-protein interaction motif. It consists of 34 amino acid residues with a loose consensus and is present in multiple tandem repeats (Das et al., 1998). Structural analysis of the TPR domain of Hop bound to the carboxyl terminus of Hsp70 or Hsp90 suggests that TPR domain has a helix-turn-helix conformation, which forms a “two-carboxylate-clmap” to hold the side chain

and carboxyl group of the terminal asparatate residue of the EEVD sequence of the carboxyl terminus of Hsp70 and Hsp90. Hydrophobic interactions with the divergent sequences amino terminal to the EEVD motif of Hsp70 or Hsp90 with the TPR domain accounts for the specificity of binding (Scheufler C, 2000). Since CHIP has three TPR repeats, the Patterson lab set out to study interactions between CHIP and Hsp70 or Hsp90 and the physiological consequences of these interactions.

CHIP contains three amino-terminal TPR repeats, a central charged domain, and a carboxyl-terminal U-box. The interaction between CHIP and Hsc70 is mediated by the amino-terminal TPR domains plus the charged domain of CHIP with the carboxyl-terminus of Hsc70, similar to the interaction between HOP and Hsp70 or Hsp90. CHIP inhibits Hsp40-stimulated ATPase activity of Hsp/Hsc70, and therefore inhibits both substrate binding and refolding activity of Hsp/Hsc70 in vitro. Thus, CHIP was implicated as a negative regulator of the Hsp/Hsc70 substrate-binding cycle (Ballinger et al., 1999).

However, overexpression of CHIP in mammalian cells in vivo increases the refolding of proteins after thermal denaturation, which is dependent on the interaction of TPR domains of CHIP with Hsp70. Hsp40 competitively inhibited the CHIP-dependent refolding, which is consistent with in vitro data indicating that these cofactors act on Hsp70 in the ATP-bound state and have opposing effects on Hsp70 ATPase activity (Kampinga et al., 2003).

To reconcile the different findings on Hsp70 refolding activity regulated by CHIP and Hsp40, we have to reconsider the model of Hsp70 nucleotide binding states and the coupled substrate binding affinity. It has been postulated that the ATP-binding state of Hsp70 has low substrate affinity which is unfavorable to folding, while the ADP-binding state of Hsp70 has high substrate affinity which favors folding. However, in the ATP-binding state, Hsp70

substrate binding domain is open, which allows Hsp70 to bind a variety of substrates. In the complicated in vivo states, a variety of cochaperones exist within cells, the relative abundance of each cochaperone and sum of their actions determines the overall refolding activity of Hsp70. Overexpressing CHIP slows down the ATPase activity of Hsp70 and allows a better loading of substrates onto Hsp70, which might be the rate-limiting step within certain cells (Kampinga et al., 2003). Therefore, it is difficult to define the endogenous role of a cochaperone in regulating Hsp70 refolding function based on in vitro data, given the complexity of the situation within the cells.

Besides the TPR domains, CHIP has a U-box domain, which is similar to the RING-finger domain of ubiquitin E3 ligase. Indeed, U-box domain of CHIP has ubiquitin E3 ligase activity, and CHIP has been suggested to be a chaperone-dependent E3 ubiquitin ligase that links the chaperone to the ubiquitin-proteasome system during protein quality control (Cyr et al., 2002; McDonough and Patterson, 2003). This function and the interaction of CHIP with Hsp90 will be discussed in the following parts of this dissertation.

1.1.1d Hip and HspBP1

Hip (Hsc70 interacting protein) was first noted as a transient component during the cell-free assembly of progesterone receptor complex (Smith et al., 1995) and was subsequently found to be associated with Hsp70 and Hsc70 (Prapapanich et al., 1996; Irmer and Hohfeld, 1997). Hip has an N-terminal homo-oligomerization domain and a central TPR repeat flanked by an acidic α-helical segment and a basic α-helix. The TPR repeats and the following α-helix mediate the binding of Hip with the ATPase domain of Hsc/Hsp70 (Hohfeld et al., 1995; Prapapanich et al., 1996; Irmer and Hohfeld, 1997; Demand et al., 1998). Hip stabilizes the ADP state of Hsc70 that has a high affinity for substrate protein

(Hohfeld et al., 1995; Irmer and Hohfeld, 1997). The amino-terminal homo-oligomerization domain does not affect the interaction between Hip and Hsp/Hsc70. It has been postulated that oligomerization of Hip fulfills a scaffolding function by holding multiple Hsc70 molecules in close proximity to an unfolded polypeptide substrate (Irmer and Hohfeld, 1997). Besides affecting the Hsc/Hsp70 chaperone activities *in vitro* and *in vivo* (Prapapanich et al., 1998; Nollen et al., 2001), Hip alone can also bind to unfolded proteins and prevent their aggregation. Yet refolding of proteins to their active state requires cooperation with other chaperones (Hohfeld et al., 1995; Prapapanich et al., 1996).

HspBP1 (heat shock protein binding protein 1) was isolated from a human heart cDNA library using the yeast two-hybrid system (Raynes and Guerriero Jr, 1998). HspBP1 binds the ATPase domain of Hsp70, induces the release of either ATP or ADP from the ATPase domain of Hsp70, and inhibits protein refolding function of Hsp70 in vitro (Raynes and Guerriero Jr, 1998; Mehdi et al., 2002). Since BAG family proteins also induce the release of ADP from the ATPase domain of Hsp70, there is functional similarity between these two proteins. However, the crystal structure of HspBP1, alone and complexed with part of the Hsp70 ATPase domain, reveals a mechanism for its function distinct from that of BAG1 or GrpE. HspBP1 has a curved, all alpha-helical fold containing four armadillo-like repeats unlike the other nucleotide exchange factors. The concave face of HspBP1 embraces lobe II of the ATPase domain of Hsp70, and a steric conflict displaces lobe I, reducing the affinity for nucleotide. In contrast, BAG-1 and GrpE trigger a conserved conformational change in lobe II of the ATPase domain. Thus, two distinct mechanisms account for nucleotide exchange on eukaryotic Hsp70 (Yasuhito et al., 2005).

1.1.2 The Hsp90 family proteins and their cochaperones

The Hsp90 chaperone family is highly conserved throughout evolution. It includes the eponymous Hsp90 (90 kD heat shock protein) of the eukaryotic cytosol, termed variously Hsp90α and β in humans (corresponding to a major and minor isoform), Hsp86 and Hsp84 in mice, Hsp83 in *Drosophila*, and Hsc82 and Hsp82 in yeast. Other family members are HtpG in the bacterial cytosol, Grp94/gp96 in the endoplasmic reticulum of eukaryotes, and the Hsp75/TRAP1 in the mitochondrial matrix (Argon and Simen, 1999; Felts et al., 2000; Thomas and Baneyx, 2000). These proteins have a common structural composition and are thus expected to have a similar mechanism of action. Hsp90 has a COOH-terminal 190-residue dimerization domain that forms constitutive Hsp90 homodimer (Nemoto et al., 1995), and a highly conserved 25 kDa NH_2-terminal domain that is the binding site for ATP and for geldanamycin (GA) (Prodromou et al., 1997; Stebbins et al., 1997). GA is a representative of the ansamycin drugs that are specific inhibitors of Hsp90 (Whitesell et al., 1994). Both the NH_2- and COOH-terminal domains of Hsp90 are able to bind to substrate polypeptides, and substrate binding at the NH_2-terminal site is affected by nucleotides, GA, and the adjacent charged sequence of Hsp90 (Young et al., 1997; Scheibel et al., 1998; Scheibel et al., 1999). Similar to the other ATP-dependent chaperones, the interaction of Hsp90 with substrate polypeptides is regulated by the ATPase cycle (Prodromou et al., 1997; Obermann et al., 1998; Panaretou et al., 1998).

Unlike Hsp70, eukaryotic cytosolic Hsp90 does not act generally in nascent protein folding (Nathan et al., 1997). Hsp90 is distinguished from other chaperones in that most of its known substrates are signal transduction proteins, the classical examples being steroid hormone receptors and signaling kinases (Picard et al., 1990; Xu and Lindquist, 1993). Because Hsp90 is essential for maintaining the activity of numerous signaling proteins, it

plays a key role in cellular signal transduction networks. A common feature of many Hsp90 clients, including steroid hormone receptors and signaling kinases, is a tendency to dwell in incompletely folded or aggregation-prone states. These proteins dynamically cycle through complexes with Hsp90 and other cofactors until their activation is engendered by the proper signal. (Jakob et al., 1995) In fulfilling its role, Hsp90 operates as part of a multichaperone machinery in the cytosol, which includes Hsp70 and other cochaperones (Bose et al., 1996; Freeman et al., 1996).

The largest class of cochaperones binds to Hsp90 via a modular domain containing typically three TPR repeats. These TPR domains are fused to a series of different functional domains, for example peptidyl-prolyl isomerase domains (the cyclophilin Cyp40 and the immunophilins FKBP52 and FKBP51) (Johnson and Toft, 1994) or a protein phosphatase (PP5) (Chen et al., 1996a), or other TPR domains that brings Hsp70 and Hsp90 into a multichaperone complex (Hop) (Chen and Smith, 1998; Johnson et al., 1998), or a U-box that diverts Hsp90 bound glucocorticoid receptor for proteasomal degradation (CHIP) The TPR containing cochaperone CHIP associates with Hsp90 containing glucocorticoid receptor-chaperone heterocomplexes and diverts chaperone substrate for proteasomal degradation (Connell et al., 2001).

In the following paragraphs I will briefly introduce Hsp90 cochaperones Hop and p23 as examples of cochaperone regulation of Hsp90 functions.

1.1.2a. Hop containing chaperone complexes

Hop (Hsp organizer protein) is composed almost exclusively of TPR domains and does not function as a chaperone on its own (Honore et al., 1992; Smith et al., 1993). Hop has nine TPR motifs that form two TPR domains. The amino-terminal TPR1 domain that contains

three TPR motifs is responsible for the interaction with the carboxyl-terminus of Hsp70, and the carboxyl-terminal TPR2 domain that contains six TPR motifs mediates the interaction of Hop with Hsp90 (Chen et al., 1996b; Lassle et al., 1997; Demand et al., 1998). Therefore Hop links the Hsp70 with the Hsp90 chaperone system. As with Hsp70, the binding site for Hop on Hsp90 is the carboxyl-terminal EEVD motif of Hsp90 (Scheufler C, 2000).

Hop inhibits the Hsp90 ATPase (Prodromou et al., 1999) and blocks access of ATP or the inhibitor geldanamycin to its binding pocket in the NH_2-terminal domain of Hsp90 (Prodromou et al., 1997; Stebbins et al., 1997; Prodromou et al., 1999). This inhibitory activity of Hop has been proposed to be part of a substrate-loading mechanism for Hsp90, where an Hsp90-Hop-Hsp70 complex permits transfer of substrate polypeptide from Hsc70 to the nucleotide-free state of Hsp90 (Prodromou et al., 1999). Binding of ATP onto Hsp90 then displaces the Hop-Hsc70 loading system and simultaneously closes the substrate-binding clamp of Hsp90. Although such a loading mechanism remains to be demonstrated directly, it is consistent with earlier time course experiments, showing that initial binding of Hsc70, Hop, and Hsp90 to progesterone receptor was followed by the dissociation of Hsc70 and Hop, leading to “mature” Hsp90 complexes (Smith et al., 1993; Hutchison et al., 1994).

1.1.2b p23 containing chaperone complexes

p23 (Sba1 in yeast), which is unrelated to the TPR domain proteins, is a small protein with chaperone activity that binds unfolded polypeptides (Bose et al., 1996; Freeman et al., 1996) and also interacts with the NH_2-terminal domain of Hsp90. The active core domain of p23 has an immunoglobulin-like fold with several highly conserved residues exposed in one cluster, forming the probable contact site for Hsp90 (Weaver et al., 2000). The core domain of p23 is sufficient for its ATP-dependent action on Hsp90 (Young and Hartl, 2000), whereas

the chaperone activity of p23 resides in a separate carboxyl terminal domain (Weikl et al., 2000).

p23 and Sba1 recognize specifically the ATP-bound state of Hsp90(Sullivan et al., 1997; Fang et al., 1998), which is present in the mature form of Hsp90 complexes. Although p23 does not affect the ATPase activity of Hsp90, it significantly stimulates the ATP hydrolysis-dependent dissociation of Hsp90-substrate complexes. One possible mechanism is that p23 more stringently couples the nucleotide state of Hsp90 with conformational changes throughout the dimer (Young and Hartl, 2000).

1.2 Heat shock response and its regulation

The heat shock response is an ordered genetic response to diverse environmental and physiological stressors that results in the immediate induction of genes encoding molecular chaperones, proteases, and other proteins essential for protection and recovery from cellular damage. The rationale behind this phenomenon is that after stress there is increased need for the chaperone function of heat shock proteins, which triggers their induction. This need is caused by the increased amount of damaged proteins, by the inhibition of their elimination via the proteasome as well as by the damage of the chaperones themselves. The induction of heat shock proteins therefore helps to bind unfolded proteins and hold them in folding-competent states for further refolding and prevent them from aggregation.

The heat shock response is regulated at the transcriptional level by the activation of a family of heat shock transcription factors (HSF) (Pirkkala et al., 2001). HSF1-4 have been identified in vertebrates and plants . Among them, HSF1 is the best characterized and essential for the heat shock response. HSF2 is important in developmentally related

conditions. HSF3 exists only in avian species. Less function has been assigned to HSF4. (Pirkkala et al., 2001; Voellmy, 2004)

1.2.1 HSF1 as the major transcription factor responsible for stress response

The DNA binding and transcriptional activities of HSF1 are stress-inducibly regulated by a multistep activation pathway. HSF exists normally in a repressed state as an inert monomer in either the cytoplasmic or nuclear compartments. Upon exposure to a variety of stresses, HSF1 is derepressed, trimerizes, and accumulates in the nucleus. HSF1 trimers bind with high affinity to the heat shock elements (HSEs) consisting of multiple contiguous inverted repeats of the pentamer sequence nGGAn located in the promoter regions of target genes (Wu, 1995). The HSF1 regulated genes encoding Hsp70, Hsp90, and small Hsps are also transcribed constitutively due to multiple basal factors or binding of low levels of HSF1.

1.2.2 Regulation of HSF1 activity

Several mechanisms have been suggested to regulate HSF1 activity, which includes the regulation by molecular chaperones Hsp70, Hsp90 and cochaperone Hsp40, posttranslational modification by phosphorylation and sumoylation, and the regulation by redox status (Zhong et al., 1998; Ahn and Thiele, 2003; Voellmy, 2004; Westerheide and Morimoto, 2005). This complicated system of the regulation of HSF1 may reflect the necessity to tightly control the expression of molecular chaperones.

1.2.2a Regulation by molecular chaperones

Both Hsp70 and Hsp90 have been implicated in the regulation of HSF1 (Morimoto, 1998). The substrate binding domain of Hsp70 interacts with the activation domain of HSF1 that features chaperone-substrate binding. This interaction has been suggested to be responsible to repress HSF1 during the attenuation phase of heat shock response. Therefore,

the induced Hsp70 exerts a feed-back control to limit the extent of heat shock response. (Abravaya et al., 1992; Shi et al., 1998)

Hsp90 has also been suggested to suppress HSF1. Hsp90-immunophilin-p23 complex interacts with the regulatory domain of HSF1 and suppresses the transcriptional activity of trimeric HSF1. During stress response when there is increased amount of denatured proteins, Hsp90-immunophilin-p23 complexes are diverted to bind to denatured proteins, or when Hsp90 or p23 are depleted by antibody in experimental conditions, HSF1 is released from the chaperone complexes, becomes trimerized and activated spontaneously (Zou et al., 1998; Guo et al., 2001). Formation of the heterocomplex may also represent the first step toward returning the HSF1 to its unactivated form.

1.2.2b Modification by phosphorylation and sumoylation

HSF1 is constitutively phosphorylated and becomes hyperphosphorylated upon stress stimuli (Cotto et al., 1996; Knauf et al., 1996; Xia and Voellmy, 1997; Chu et al., 1998). Sites of HSF1 constitutive phosphorylation, including serines 303, 307 and 308, appear to be important for the negative regulation of HSF1, whereas sites of inducible phosphorylation, including serines 230, 326 and 419, promote HSF1 activity (Holmberg et al., 2001; Guettouche et al., 2005; Kim et al., 2005). The balance of kinase and phosphatase activities acting on HSF1 is of fundamental importance to regulation of the heat shock response, as suggested by mathematical modeling (Rieger et al., 2005).

SUMO (Small ubiquitin-like modifier) is a protein of 97 amino acids that is structurally similar to ubiquitin (Melchior, 2000). Like ubiquitin, SUMO has been found to be covalently attached to certain lysine residues of specific target proteins (Melchior, 2000). In contrast to ubiquitylation, however, sumoylation does not promote the degradation of proteins but

instead alters a number of different functional parameters of proteins, such as subcellular localization, protein partnering, and DNA-binding and/or transactivation functions of transcription factors (Hay, 2005). While HSF1 is sumoylated on lysine 298 in a manner that requires phosphorylation on serines 303 and 307 (Hong et al., 2001; Hietakangas et al., 2003), the role of this modification on HSF1 activity is still unclear.

1.2.2c Redox regulation of HSF1

Both heat and hydrogen peroxide can induce trimerization and DNA binding of recombinant Drosophila or mammalian HSF1 directly and reversibly in vitro (Zhong et al., 1998; Ahn and Thiele, 2003). Further examination of the mammalian HSF1 elucidated that two cysteine residues within the HSF1 DNA-binding domain are required to sense both stresses and are engaged in redox-sensitive disulfide bonds. Mutations in either or both of the cysteine residues lead to defects in stress-inducible trimerization and DNA binding, stress-inducible nuclear translocation and Hsp gene transactivation, and in the protection of mouse cells from stress-induced apoptosis (Ahn and Thiele, 2003). Thus, the redox-dependent activation of HSF1 by heat and hydrogen peroxide establishes a common mechanism in the stress activation of Hsp gene expression by HSF1.

1.3 The ubiquitin-proteasome system

1.3.1 Discovery of the protein degradation system

Tight control of protein synthesis, activity and removal from the cellular repertory is important to maintain homeostasis. When cellular proteins are damaged or unnecessary, they need to be removed. Timing and fine control of protein degradation is equally as important as the control of gene expression. Originally thought to be carried out by cellular proteases by simple chemical catalysis, now it is well-established that protein degradation requires the

complicated ubiquitin-proteasome system (Glickman and Ciechanover, 2002). In 1984, Ciechanover and Finley (Ciechanover et al., 1984; Finley et al., 1984) discovered that in the temperature sensitive ts85 cells, a mutation of the ubiquitin activating enzyme E1 caused a marked defect of ubiquitin conjugation in cells grown in non-permissive temperature compared with cells grown in permissive temperature. Importantly, turnover of abnormal or truncated polypeptides was inhibited by more than 80%, and turnover of short-lived protein was inhibited by more than 90% when cells were grown in non-permissive temperature. This discovery provided a cause and effect relationship between protein ubiquitylation and degradation for the first time. After exploration of this system for two decades, we now know more details about this system.

1.3.2 Players of the system

1.3.2a Ubiquitin

Ubiquitin is a highly conserved 76 amino acid polypeptide that can be joined to a substrate via an isopeptide bond of the carboxyl terminal glycine of ubiquitin with the lysine of its substrate in the presence of ATP. Once an ubiquitin molecule is added onto a substrate, a subsequent polyubiquitin chain is formed by isopeptide bond between carboxyl terminus of one ubiquitin and lysine residue of the previously conjugated ubiquitin. Most polyubiquitin chains form via a lysine (K) 48 link, which usually leads to substrate degradation. Other links, including K11, K29 and K63 are also present in vivo. K63-linked chains are believed to act as signaling molecules in diverse cellular pathways including endocytosis, stress response, and DNA repair (Spence et al., 1995; Soetens et al., 2001; Wang et al., 2001). Less is known about the K11 or K29 link.

1.3.2b E1, E2, E3

Protein ubiquitylation is a sequential enzymatic reaction requiring the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme, and the E3 ubiquitin ligase (Pickart, 2001; Glickman and Ciechanover, 2002). E1 catalyzes the activation of ubiquitin by forming a thioester bond between its cysteine residue and the carboxyl terminal glycine of ubiquitin at the expense of ATP. The activated ubiquitin is then transferred to a cysteine residue of an E2 ubiquitin conjugation enzyme, and further to the substrate in an E3 ubiquitin ligase dependent manner (Hershko et al., 1983). To date one E1, dozens of E2s and hundreds of E3s have been identified in mammalian cells, which is consistent with substrate specificity conferred by E3. There are two canonical types of E3 enzymes, i.e., the HECT (Homologous to E6-AP Carboxyl Terminus) domain E3s (Huibregtse et al., 1993; Huibregtse et al., 1995) and the RING (Really Interesting New Gene) finger domain E3s (Tyers and Willems, 1999; Joazeiro and Weissman, 2000). Recently the U-box proteins joined the E3 family (Murata et al., 2001; Cyr et al., 2002; Patterson, 2002).

All E3 enzymes possess two features: interaction with specific E2 and recognition of their cognate substrates. The HECT domain E3s are “true” E3 ligases because they pass ubiquitin from E2s to their cysteine residues and then on to their substrates (Scheffner et al., 1995). The structure of HECT domain E3s, which is highly conserved, is modular: The unique NH_2-terminus of each family interacts with specific substrates, and the COOH-terminal HECT domain mediates E2 binding and catalysis of ubiquitin chain formation (Huang et al., 1999). A representative of this family E3s is E6-AP (E6-associated protein). E6-AP forms complex with HPV viral protein E6 to ubiquitylate p53 (Scheffner et al., 1993). Several other members of this family, including Smurf1, Itch, and hrFP1/Nedd4 have been

identified which catalyze polyubiquitin chain formation in similar mechanism (Pickart, 2001).

The RING finger family E3s is characterized by harboring a series of histidine and cysteine residues with characteristic spacing that allows for the coordination of two zinc ions in a cross-brace structure called a RING finger (Saurin et al., 1996; Borden, 2000). The zinc bound residues are catalytic inert, and it is the spacing of the zinc ligand, rather than the sequence in between these ligand residues, that is conserved in the U-box family (Aravind and Koonin, 2000). These features suggest that the RING finger is a scaffold motif rather than a substrate recognition domain or an E3 ligase per se (Borden, 2000). The RING finger domain interacts with E2 and other domains of the RING finger protein or proteins complexed with it in order to recognize the substrate. RING finger domain E3s do not have active cysteine residues to form E3-ubiquitin intermediate as the HECT domain E3s do, rather they facilitate the transfer of ubiquitin from E2s to their substrates (Freemont, 2000). However, the precise mechanism of the catalysis is not clear. The RING finger domain-containing E3 family is composed of two distinct groups, single subunit and multisubunit proteins. Single subunit proteins are monomers or homodimers and contain both the RING finger domain and the substrate-binding/recognition site in the same molecule. Examples of single subunit E3s are c-Cbl, Mdm2, and Parkin with their substrates of EGF and PDGF receptor, p53, and Pael receptor respectively (Joazeiro et al., 1999; Boyd et al., 2000; Geyer et al., 2000; Shimura et al., 2000). Multisubunit RING E3s are classified into three subfamilies: SCF (Skp1-Cullin-F-box protein) complexes, the APC (Anaphase Promoting Complex) E3s, and the VCB (von-Hippel-Lindau-Elongins B and C) E3s. These complexes have been reviewed extensively (Pickart, 2001; Jackson and Eldridge, 2002; Peters, 2003);

here I will briefly introduce the SCF complex as an example. In this complex, the F-box protein recognizes the substrates, Skip and Cullin act as adaptors to bring the other components of the complex together. Rbx1 (Hrt1/Roc1), the RING finger domain protein, brings ubiquitylated E2 and acts as a scaffold protein (Kamura et al., 1999; Skowyra et al., 1999). The SCF complexes and the APC complexes play important roles in cell cycle control as they degrade specific cyclins that control the activity of CDKs.

Recently the U-box family proteins were identified as a new family of E3 ubiquitin ligases (Patterson, 2002; Hatakeyama and Nakayama, 2003). The U-box forms a structure similar to the RING finger domain. However, it does not have the conserved histidine and cysteine residues required to stabilize the RING finger structure. Instead the U-box structure is stabilized by a more decentralized set of hydrogen-bonding and ionic-bridging interactions than that provided by the zinc ions in the RING finger domain (Ohi et al., 2003). Given the structural similarity of U-box with RING finger domain, it was inferred that the U-box has E3 ligase activity. Yeast Ufd2, the first member of this family of proteins identified, was defined as an E4 polyubiquitin chain assembly factor. Ufd2 can not catalyze polyubiquitin chain formation on its substrate de novo. However, it is required for efficient polyubiquitin chain formation with Ufd4 (a yeast HECT domain E3) and to trigger proteasomal degradation of artificial substrates (Koegl et al., 1999). CHIP, which was originally identified as a negative regulator of Hsc/HSP70 molecular chaperones via the interaction of the TPR domain of CHIP to the carboxyl terminal EEVD motif of chaperone (Ballinger et al., 1999), has been identified as a U-box dependent E3 ubiquitin ligase (Connell et al., 2001; Jiang et al., 2001; Meacham et al., 2001). In addition to CHIP, several other U-box proteins, i.e., UIP5, CYC4, and PRP19, have also been shown to possess E3 ligase activity in the absence

of other E3 components, proving that U-box proteins are a new family of E3 ubiquitin ligase (Hatakeyama and Nakayama, 2003).

1.3.2c The proteasome

Multiple substrate-linked polyubiquitin chains signal the recruitment of the proteasome-the 2.5 MDa degradation machinery. The proteasome is composed of the 19S regulatory particle and the 20S core particle. The 19S regulatory particle recognizes polyubiquitylated substrates (Thrower et al., 2000), removes ubiquitin chain from the substrates (Eytan et al., 1993; Lam et al., 1997; Holzl et al., 2000), unfolds and translocates the substrates to the 20S core proteasome, where the substrates are degraded into small peptides (Lam et al., 1997). Polyubiquitin chain removal, substrate unfolding and translocation must be precisely coordinated in order for successful degradation of the substrate, which has been reviewed previously (Pickart and Cohen, 2004).

1.4 Functions of CHIP in protein quality control

CHIP was originally identified as a TPR domain containing cochaperone that interacts with Hsp/Hsc70 and negatively regulate Hsp/Hsc70 chaperone function (Ballinger et al., 1999). Subsequently CHIP was suggested to induce the degradation of Hsp90 client glucocorticoid receptor (GR) and chaperone associated, inefficiently folded forms of CFTR (Connell et al., 2001; Meacham et al., 2001). The direct evidence that the U-box of CHIP contains E3 ubiquitin ligase activity came from the in vitro reconstitution of polyubiquitin chain formation catalyzed by CHIP and the identification of UBC4 and UBCH5 as the E2s that couple with CHIP (Jiang et al., 2001; Murata et al., 2001). Although the function of Hsc70 ubiquitylation has unknown function (Jiang et al., 2001), the fact that CHIP ubiquitylates heat denatured luciferase that is captured by chaperones Hsp90 or Hsp70 and

Hsp40, with the previous findings that CHIP degrades GR and CFTR, suggests that CHIP is a quality-control E3 that selectively ubiquitylates unfolded or misfolded chaperone substrates, thus provides a link between the chaperone and the proteasome system.

Global suppression of chaperone function and diversion of chaperone substrate to the degradation pathway by CHIP are unfavorable for the cells to survive stress challenges as well as to maintain function in normal growth conditions. In chapter two, we studied the endogenous function of CHIP during stress conditions and found that CHIP confers protection against apoptosis and cellular stress by activating HSF1 and increasing the expression of Hsp70. In Chapter three, we examined CHIP-containing complexes and found that BAG2, a previously uncharacterized BAG domain protein, is a major component of these complexes. BAG2 suppresses CHIP-dependent E3 ligase activity specifically and efficiently. Thus, our work provides addition information about the function and regulation of CHIP in protein quality control.

1.5 References

Abravaya K, Myers M, Murphy S and Morimoto R (1992) The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes Dev* **6**:1153-1164.

Ahn S-G and Thiele DJ (2003) Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. *Genes Dev.* **17**:516-528.

Anfinsen CB (1973) Principles that govern the folding of protein chains. *Science* **181**:223-230.

Aravind L and Koonin E (2000) The U box is a modified RING finger - a common domain in ubiquitination. *Curr Biol* **10**:R132-134.

Argon Y and Simen B (1999) GRP94, an ER chaperone with protein and peptide binding properties. *Semin Cell Dev Biol.* **10**:495-505.

Ballinger CA, Connell P, Wu Y, Hu Z, Thompson LJ, Yin L-Y and Patterson C (1999) Identification of CHIP, a Novel Tetratricopeptide Repeat-Containing Protein That Interacts with Heat Shock Proteins and Negatively Regulates Chaperone Functions. *Mol. Cell. Biol.* **19**:4535-4545.

Borden KLB (2000) RING domains: master builders of molecular scaffolds? *Journal of Molecular Biology* **295**:1103-1112.

Bose S, Weikl T, Bugl H and Buchner J (1996) Chaperone Function of Hsp90-Associated Proteins. *Science* **274**:1715-1717.

Boyd S, Tsai K and Jacks T (2000) An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. *Nat Cell Biol.* **2**:563-568.

Brehmer D, Rudiger S, Gassler C, Klostermeier D, Packschies L, Reinstein J, Mayer M and Bukau B (2001) Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. *Nat Struct Biol.* **8**:427-432.

Briknarova K, Takayama S, Brive L, Havert M, Knee D, Velasco J, Homma S, Cabezas E, Stuart J, Hoyt D, Satterthwait A, Llinas M, Reed J and Ely K (2001) Structural analysis of BAG1 cochaperone and its interactions with Hsc70 heat shock protein. *Nat Struct Biol.* **8**:349-352.

Bukau B and Horwich A (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* **92**:351-366.

Chen M-S, Silverstein AM, Pratt WB and Chinkers M (1996a) The Tetratricopeptide Repeat Domain of Protein Phosphatase 5Mediates Binding to Glucocorticoid Receptor

Heterocomplexes and Acts as a Dominant Negative Mutant. *J. Biol. Chem.* **271**:32315-32320.

Chen S, Prapapanich V, Rimerman R, Honore B and Smith D (1996b) Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. *Mol Endocrinol* **10**:682-693.

Chen S and Smith DF (1998) Hop as an Adaptor in the Heat Shock Protein 70 (Hsp70) and Hsp90 Chaperone Machinery. *J. Biol. Chem.* **273**:35194-35200.

Chu B, Zhong R, Soncin F, Stevenson MA and Calderwood SK (1998) Transcriptional activity of heat shock factor 1 at 37 degrees C is repressed through phosphorylation on two distinct serine residues by glycogen synthase kinase 3 and protein kinases Calpha and Czeta. *J Biol Chem* **273**:18640-18646.

Ciechanover A, Finley D and Varshavsky A (1984) Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. *Cell* **37**:57-66.

Connell P, Ballinger CA, Jiang J, Wu Y and Patterson C (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol.* **3**:93-96.

Cotto JJ, Kline M and Morimoto RI (1996) Activation of Heat Shock Factor 1 DNA Binding Precedes Stress-induced Serine Phosphorylation. *J. Biol. Chem.* **271**:3355-3358.

Cyr DM, Hohfeld J and Patterson C (2002) Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. *Trends in Biochemical Sciences* **27**:368-375.

Das A, Cohen P and Barford D (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *Embo J* **17**:1192-1199.

Demand J, Luders J and Hohfeld J (1998) The Carboxy-Terminal Domain of Hsc70 Provides Binding Sites for a Distinct Set of Chaperone Cofactors. *Mol. Cell. Biol.* **18**:2023-2028.

Ehrnsperger M, Graber S, Gaestel M and Buchner J (1997) Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *EMBO J.* **16**:221-229.

Eytan E, Armon T, Heller H, Beck S and Hershko A (1993) Ubiquitin C-terminal hydrolase activity associated with the 26 S protease complex. *J. Biol. Chem.* **268**:4668-4674.

Fang Y, Fliss AE, Rao J and Caplan AJ (1998) SBA1 Encodes a Yeast Hsp90 Cochaperone That Is Homologous to Vertebrate p23 Proteins. *Mol. Cell. Biol.* **18**:3727-3734.

Felts SJ, Owen BAL, Nguyen P, Trepel J, Donner DB and Toft DO (2000) The hsp90-related Protein TRAP1 Is a Mitochondrial Protein with Distinct Functional Properties. *J. Biol. Chem.* **275**:3305-3312.

Finley D, Ciechanover A and Varshavsky A (1984) Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. *Cell* **37**:43-55.

Freeman BC, Toft DO and Morimoto RI (1996) Molecular Chaperone Machines: Chaperone Activities of the Cyclophilin Cyp-40 and the Steroid Aporeceptor-Associated Protein p23. *Science* **274**:1718-1720.

Freemont P (2000) RING for destruction? *Curr Biol* **10**:R84-87.

Geyer R, Yu Z and Maki C (2000) The MDM2 RING-finger domain is required to promote p53 nuclear export. *Nat Cell Biol.* **2**:569-573.

Glickman MH and Ciechanover A (2002) The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol. Rev.* **82**:373-428.

Guettouche T, Boellmann F, Lane W and Voellmy R (2005) Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochem* **6**:4.

Guo Y, Guettouche T, Fenna M, Boellmann F, Pratt WB, Toft DO, Smith DF and Voellmy R (2001) Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex. *J. Biol. Chem.*:M105931200.

Hartl FU and Hayer-Hartl M (2002) Molecular Chaperones in the Cytosol: from Nascent Chain to Folded Protein. *Science* **295**:1852-1858.

Hatakeyama S and Nakayama K-iI (2003) U-box proteins as a new family of ubiquitin ligases. *Biochemical and Biophysical Research Communications* **302**:635-645.

Hay R (2005) SUMO: a history of modification. *Mol Cell* **18**:1-12.

Hershko A, Heller H, Elias S and Ciechanover A (1983) Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J. Biol. Chem.* **258**:8206-8214.

Hietakangas V, Ahlskog JK, Jakobsson AM, Hellesuo M, Sahlberg NM, Holmberg CI, Mikhailov A, Palvimo JJ, Pirkkala L and Sistonen L (2003) Phosphorylation of Serine 303 Is a Prerequisite for the Stress-Inducible SUMO Modification of Heat Shock Factor 1. *Mol. Cell. Biol.* **23**:2953-2968.

Hohfeld J, Minami Y and Hartl F (1995) Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. *Cell* **83**:589-598.

Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M, Meinander A, Hellman J, Morrice N, MacKintosh C, Morimoto RI, Eriksson JE and Sistonen L (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *Embo J* **20**:3800-3810.

Holzl H, Kapelari B, Kellermann J, Seemuller E, Sumegi M, Udvardy A, Medalia O, Sperling J, Muller SA, Engel A and Baumeister W (2000) The Regulatory Complex of Drosophila melanogaster 26S Proteasomes: Subunit Composition and Localization of a Deubiquitylating Enzyme. *J. Cell Biol.* **150**:119-130.

Hong Y, Rogers R, Matunis MJ, Mayhew CN, Goodson M, Park-Sarge OK and Sarge KD (2001) Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J Biol Chem* **276**:40263-40267.

Honore B, Leffers H, Madsen P, Rasmussen HH, Vandekerckhove J and Celis JE (1992) Molecular cloning and expression of a transformation-sensitive human protein containing the TPR motif and sharing identity to the stress- inducible yeast protein STI1. *J. Biol. Chem.* **267**:8485-8491.

Horwich AL, Weber-Ban EU and Finley D (1999) Chaperone rings in protein folding and degradation. *Proc Natl Acad Sci U S A.* **96**:11033-11040.

Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM and Pavletich NP (1999) Structure of an E6AP-UbcH7 Complex: Insights into Ubiquitination by the E2-E3 Enzyme Cascade. *Science* **286**:1321-1326.

Huibregtse J, Scheffner M and Howley P (1993) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *mol Cell Biol* **13**:775-784.

Huibregtse JM, Scheffner M, Beaudenon S and Howley PM (1995) Correction: A Family of Proteins Structurally and Functionally Related to the E6-AP Ubiquitin-Protein Ligase. *PNAS* **92**:5249a-.

Hutchison KA, Dittmar KD, Czar MJ and Pratt WB (1994) Proof that hsp70 is required for assembly of the glucocorticoid receptor into a heterocomplex with hsp90. *J. Biol. Chem.* **269**:5043-5049.

Irmer H and Hohfeld J (1997) Characterization of Functional Domains of the Eukaryotic Co-chaperone Hip. *J. Biol. Chem.* **272**:2230-2235.

Jackson PK and Eldridge AG (2002) The SCF ubiquitin ligase: an extended look. *Mol Cell.* **9**:923-925.

Jakob U, Lilie H, Meyer I and Buchner J (1995) Transient Interaction of Hsp90 with Early Unfolding Intermediates of Citrate Synthase. *J. Biol. Chem.* **270**:7288-7294.

Jiang J, Ballinger CA, Wu Y, Dai Q, Cyr DM, Hohfeld J and Patterson C (2001) CHIP Is a U-box-dependent E3 Ubiquitin Ligase. IDENTIFICATION OF Hsc70 AS A TARGET FOR UBIQUITYLATION. *J. Biol. Chem.* **276**:42938-42944.

Joazeiro C and Weissman A (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**:549-552.

Joazeiro CA, nbsp, P, Wing SS, Huang H-k, Leverson JD, Hunter T and Liu Y-C (1999) The Tyrosine Kinase Negative Regulator c-Cbl as a RING-Type, E2-Dependent Ubiquitin-Protein Ligase. *Science* **286**:309-312.

Johnson BD, Schumacher RJ, Ross ED and Toft DO (1998) Hop Modulates hsp70/hsp90 Interactions in Protein Folding. *J. Biol. Chem.* **273**:3679-3686.

Johnson JL and Toft DO (1994) A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. *J. Biol. Chem.* **269**:24989-24993.

Kampinga HH, Kanon B, Salomons FA, Kabakov AE and Patterson C (2003) Overexpression of the Cochaperone CHIP Enhances Hsp70-Dependent Folding Activity in Mammalian Cells. *Mol. Cell. Biol.* **23**:4948-4958.

Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, Iliopoulos O, Lane WS, Kaelin WG, Jr., Elledge SJ, Conaway RC, Harper JW and Conaway JW (1999) Rbx1, a Component of the VHL Tumor Suppressor Complex and SCF Ubiquitin Ligase. *Science* **284**:657-661.

Kelley W (1998) The J-domain family and the recruitment of chaperone power. *Trends Biochem Sci.* **6**:222-227.

Kim S-A, Yoon J-H, Lee S-H and Ahn S-G (2005) Polo-like Kinase 1 Phosphorylates Heat Shock Transcription Factor 1 and Mediates Its Nuclear Translocation during Heat Stress. *J. Biol. Chem.* **280**:12653-12657.

Knauf U, Newton E, Kyriakis J and Kingston R (1996) Repression of human heat shock factor 1 activity at control temperature by phosphorylation. *Gene Dev.* **10**:2782-2793.

Koegl M, Hoppe T, Schlenker S, Ulrich HD, Mayer TU and Jentsch S (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *cell* **96**:635-644.

Lam Y, Xu W, DeMartino G and Cohen R (1997) Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature* **385**.

Lassle M, Blatch GL, Kundra V, Takatori T and Zetter BR (1997) Stress-inducible, Murine Protein mSTI1. CHARACTERIZATION OF BINDING DOMAINS FOR HEAT

SHOCK PROTEINS AND IN VITRO PHOSPHORYLATION BY DIFFERENT KINASES. *J. Biol. Chem.* **272**:1876-1884.

Mayer MP, Schröder H, Rüdiger S, Paal K, Laufen T and Bukau B (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat Struct Biol.* **7**:586-593.

McDonough H and Patterson C (2003) CHIP: a link between the chaperone and proteasome systems. *cell Stress Chaperones* **8**:303-308.

Meacham G, Lu Z, King S, Sorscher E, Tousson A and Cyr D (1999) The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. **18**:1492-1501.

Meacham GC, Patterson C, Zhang W, Younger JM and Cyr DM (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol.* **3**:100-105.

Mehdi K, Catherine M, A. RD, Guerrierob Vc and L. BJ (2002) HspBP1, a homologue of the yeast Fes1 and Sls1 proteins, is an Hsc70 nucleotide exchange factor. *FEBS Letters* **531**:339-342.

Melchior F (2000) SUMO--nonclassical ubiquitin. *Annu Rev Cell Dev Biol* **16**:591-626.

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* **12**:3788-3796.

Murata S, Minami Y, Minami M, Chiba T and Tanaka K (2001) CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.* **2**.

Nathan DF, Vos MH and Lindquist S (1997) In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. *PNAS* **94**:12949-12956.

Nemoto T, Ohara-Nemoto Y, Takagi T and Yokoyama K (1995) Mechanism of dimer formation of the 90-kDa heat-shock protein. *Eur J Biochem* **233**:1-8.

Nollen EAA, Kabakov AE, Brunsting JF, Kanon B, Hohfeld J and Kampinga HH (2001) Modulation of in Vivo HSP70 Chaperone Activity by Hip and Bag-1. *J. Biol. Chem.* **276**:4677-4682.

Obermann WMJ, Sondermann H, Russo AA, Pavletich NP and Hartl FU (1998) In Vivo Function of Hsp90 Is Dependent on ATP Binding and ATP Hydrolysis. *J. Cell Biol.* **143**:901-910.

Ohi MD, Vander KCW, Rosenberg JA, Chazin WJ and Gould KL (2003) Structural insights into the U-box, a domain associated with multi-ubiquitination. *Nat Struct Biol.* **10**:250-255.

Panaretou B, Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW and Pearl LH (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J.* **17**:4829-4836.

Patterson C (2002) A new gun in town: the U box is a ubiquitin ligase domain. *Sci STKE* **116**:PE4.

Pellecchia M, Montgomery DL, Stevens SY, Kooi CWV, Feng H-p, Gierasch LM and Zuiderweg ERP (2000) Structural insights into substrate binding by the molecular chaperone DnaK. *Nat Struct Biol.* **7**:298-303.

Peters JM (2003) The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol Cell* **9**:931-943.

Picard D, Khursheed B, Garabedian MJ, Fortin MG, Lindquist S and Yamamoto KR (1990) Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* **348**:166–168.

Pickart CM (2001) Mechanisms undelying ubiquitination. *Annual Review of Biochemistry* **70**:503-533.

Pickart CM and Cohen RE (2004) Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol.* **5**:177-187.

Pirkkala L, Nykanen P and Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.* **15**:1118-1131.

Prapapanich V, Chen S, Nair SC, Rimerman RA and Smith DF (1996) Molecular cloning of human p48, a transient component of progesterone receptor complexes and an Hsp70-binding protein. *Mol Endocrinol* **10**:420-431.

Prapapanich V, Chen S and Smith DF (1998) Mutation of Hip's Carboxy-Terminal Region Inhibits a Transitional Stage of Progesterone Receptor Assembly. *Mol. Cell. Biol.* **18**:944-952.

Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW and Pearl LH (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90**:65–75.

Prodromou C, Siligardi G, O'Brien R, Woolfson D, Regan L, Panaretou B, Ladbury J, Piper P and Pearl L (1999) Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. *Embo J* **18**:754-762.

Raynes DA and Guerriero Jr V (1998) Inhibition of Hsp70 ATPase Activity and Protein Renaturation by a Novel Hsp70-binding Protein. *J. Biol. Chem.* **273**:32883-32888.

Rieger TR, Morimoto RI and Hatzimanikatis V (2005) Mathematical Modeling of the Eukaryotic Heat-Shock Response: Dynamics of the hsp70 Promoter. *Biophys. J.* **88**:1646-1658.

Rudiger S, Schneider-Mergener J and Bukau B (2001) Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *EMBO J.* **20**:1042-1050.

Saurin A, Borden K, Boddy M and Freemont P (1996) Does this have a familiar RING? *Trends Biochem Sci.* **21**:208-214.

Scheffner M, Huibregtse JM, Vierstra RD and Howley PM (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *cell* **75**:495-505.

Scheffner M, Nuber U and Huibregtse JM (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**:81.

Scheibel T, Siegmund HI, Jaenicke R, Ganz P, Lilie H and Buchner J (1999) The charged region of Hsp90 modulates the function of the N-terminal domain. *PNAS* **96**:1297-1302.

Scheibel T, Weikl T and Buchner J (1998) Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. *PNAS* **95**:1495-1499.

Scheufler C BA, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *cell* **101**:199-210.

Sha B, Lee S and Cyr D (2000) The crystal structure of the peptide-binding fragment from the yeast Hsp40 protein Sis1. *Structure Fold Des* **8**:799-807.

Shi Y, Mosser DD and Morimoto RI (1998) Molecularchaperones as HSF1-specific transcriptional repressors. *Genes Dev.* **12**:654-666.

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K and Suzuki T (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet.* **25**:302-305.

Skowyra D, Koepp DM, Kamura T, Conrad MN, Conaway RC, Conaway JW, Elledge SJ and Harper JW (1999) Reconstitution of G1 Cyclin Ubiquitination with Complexes Containing SCFGrr1 and Rbx1. *Science* **284**:662-665.

Smith D, Sullivan W, Marion T, Zaitsu K, Madden B, McCormick D and Toft D (1993) Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol Cell Biol.* **13**:869-876.

Smith DF, Whitesell L, Nair SC, Chen S, Prapapanich V and Rimerman RA (1995) Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol.* **15**:6804-6812.

Soetens O, De Craene J-O and Andre B (2001) Ubiquitin Is Required for Sorting to the Vacuole of the Yeast General Amino Acid Permease, Gap1. *J. Biol. Chem.* **276**:43949-43957.

Sondermann H, Scheufler C, Schneider C, Hohfeld J, Hartl F and Moarefi I (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science* **291**:1553-1557.

Spence J, Sadis S, Haas A and Finley D (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* **15**:1265-1273.

Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU and Pavletich NP (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* **89**:239–250.

Sullivan W, Stensgard B, Caucutt G, Bartha B, McMahon N, Alnemri ES, Litwack G and Toft D (1997) Nucleotides and Two Functional States of hsp90. *J. Biol. Chem.* **272**:8007-8012.

Takayama S and Reed J (2001) Molecular chaperone targeting and regulation by BAG family proteins. *Nat Cell Biol.* **3**:E237-241.

Takayama S, Xie Z and Reed JC (1999) An Evolutionarily Conserved Family of Hsp70/Hsc70 Molecular Chaperone Regulators. *J. Biol. Chem.* **274**:781-786.

Thomas JG and Baneyx F (2000) ClpB and HtpG facilitate de novo protein folding in stressed Escherichia coli cells. *Molecular Microbiology* **36**:1360-1370.

Thrower JS, Hoffman L, Rechsteiner M and Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19**:94-102.

Tyers M and Willems A (1999) One ring to rule a superfamily of E3 ubiquitin ligases. *Science* **284**:601-604.

Voellmy R (2004) On mechanisms that control heat shock transcription factor activity in metazoan cells. *Cell Stress Chaperones* **9**:122-133.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J and Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**:346-351.

Weaver AJ, Sullivan WP, Felts SJ, Owen BAL and Toft DO (2000) Crystal Structure and Activity of Human p23, a Heat Shock Protein 90 Co-chaperone. *J. Biol. Chem.* **275**:23045-23052.

Weikl T, Muschler P, Richter K, Veit T, Reinstein J and Buchner J (2000) C-terminal regions of Hsp90 are important for trapping the nucleotide during the ATPase cycle. **303**:583-592.

Westerheide SD and Morimoto RI (2005) Heat Shock Response Modulators as Therapeutic Tools for Diseases of Protein Conformation. *J. Biol. Chem.* **280**:33097-33100.

Whitesell L, Mimnaugh EG, De Costa B, Myers CE and Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci U S A* **91**:8324–8328.

Wickner S, Maurizi MR and Gottesman S (1999) Posttranslational Quality Control: Folding, Refolding, and Degrading Proteins. *Science* **286**:1888-1893.

Wu C (1995) Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* **11**:441-469.

Xia W and Voellmy R (1997) Hyperphosphorylation of Heat Shock Transcription Factor 1Is Correlated with Transcriptional Competence and Slow Dissociation of Active Factor Trimers. *J. Biol. Chem.* **272**:4094-4102.

Xu Y and Lindquist S (1993) Heat-Shock Protein hsp90 Governs the Activity of pp60v-src Kinase. *PNAS* **90**:7074-7078.

Yasuhito S, Zdravko D, Hung-Chun C, Nikolay T, C. YJ, L. BJ, Guerriero Vince F, Ulrich H and Andreas B (2005) Regulation of Hsp70 Function by HspBP1 Structural Analysis Reveals an Alternate Mechanism for Hsp70 Nucleotide Exchange. *Mol Cell* **17**:367-379.

Young JC, Agashe VR, Siegers K and Hartl FU (2004) Pathways of chaperone-mediated protein folding in the cytosol. *Nat Rev Mol Cell Biol.* **5**:781-791.

Young JC and Hartl FU (2000) Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. *EMBO J.* **19**:5930-5940.

Young JC, Schneider C and Hartl FU (1997) In vitro evidence that hsp90 contains two independent chaperone sites. *FEBS Letters* **418**:139.

Zhong M, Orosz A and Wu C (1998) Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. *Mol Cell.* **2**:101-108.

Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman ME and Hendrickson WA (1996) Structural Analysis of Substrate Binding by the Molecular Chaperone DnaK. *Science* **272**:1606-1614.

Zou J, Guo Y, Guettouche T, Smith D and Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *cell* **94**:471-480.

Chapter 2

CHIP activates HSF1 and confers protection against apoptosis and cellular stress

Part of this chapter was published as: Dai Q, Zhang C, Wu Y, McDonough H, Whaley RA, Godfrey V, Li H-H, Madamanchi N, Xu W, Neckers L, Cyr D and Patterson C (2003) CHIP activates HSF1 and confers protection against apoptosis and cellular stress. *EMBO J.* 22:5446-5458.

2.1 Abstract

Induction of molecular chaperones is the characteristic protective response to environmental stress, and is regulated by a transcriptional program that depends on heat shock factor 1 (HSF1), which is normally under negative regulatory control by molecular chaperones Hsp70 and Hsp90. In metazoan species, the chaperone system also provides protection against apoptosis. We demonstrate that the dual function co-chaperone/ubiquitin ligase CHIP (C-terminus of Hsp70-interacting protein) regulates activation of the stress-chaperone response through induced trimerization and transcriptional activation of HSF1, and is required for protection against stress-induced apoptosis in murine fibroblasts. CHIP exerts a central and unique role in tuning the response to stress at multiple levels by regulation of protein quality control and transcriptional activation of stress response signaling.

2.2 Introduction

Protection from cellular stress is a fundamental function that enables all living organisms to counteract noxious environmental stimuli. A crucial aspect of the heat shock response is the rapid and massive production of distinct classes of related proteins conserved in evolution, including heat shock proteins 70 and 90, which can account for several percent of all proteins within a cell in times of stress. Although originally appreciated as a mechanism for protection against thermal stress, the so-called heat shock response is activated by a range of other stressors (including osmotic changes, ischemia and aging) all of which have the accumulation of misfolded proteins as a central feature. Heat shock proteins act as molecular chaperones that bind to misfolded proteins, prevent their aggregation and aid their refolding. In addition, functions distinct from their chaperone activity have emerged. Both Hsp70 and Hsp90 assist in the delivery of fatally damaged proteins to the ubiquitin–proteasome protein degradation machinery and modulate the apoptotic response (Schneider et al., 1996; Bercovich et al., 1997; Gabai et al., 1997; Mosser et al., 1997; Beere and Green, 2001); therefore, the overall function of heat shock proteins is to promote cellular survival by providing a protected environment within the cell until non-native proteins can be folded or cleared.

Expression of heat shock proteins is tightly controlled at the transcriptional level through regulation of heat shock factor 1 (HSF1). HSF1 is repressed by Hsp90, Hsp70 and HDJ1 (Hsp40) during quiescent state (Shi et al., 1998; Zou et al., 1998). Upon activation, HSF1 becomes trimerized, acquires posttranslational modification such as phosphorylation, and is translocated into the nucleus, where it binds to heat shock elements (HSEs) located in the promoters of heat shock proteins (Morimoto, 1998). Molecular chaperones, including

Hsp70, Hsp90 and HDJ1 (Hsp40), also play a role in the attenuation phase of HSF1 activation (Rabindran et al., 1994; Shi et al., 1998; Zou et al., 1998). In particular, Hsp70 interacts with the transactivation domain of HSF1 and attenuates HSF1 activity, in part by preventing recruitment of the general transcriptional machinery and also by stabilizing the monomeric conformation (Shi et al., 1998). Repression of HSF1 activity by stress-inducible chaperones forms a feedback regulation of the HSF1 activation pathway, which is under tight control at multiple levels (Zuo et al., 1995).

Activities of molecular chaperones of Hsp70 and Hsp90 are controlled by families of accessory proteins called co-chaperones [reviewed in (Luders et al., 1998)]. For Hsp70, these co-chaperones regulate cycles of ATP binding, hydrolysis into ADP and nucleotide exchange of ADP with ATP, which in turn control substrate affinity and folding activity. One such co-chaperone, CHIP (C-terminus of Hsp70-interacting protein), interacts with Hsp70 (and also Hsp90) via three tandem tetratricopeptide repeat (TPR) motifs. CHIP, which is highly conserved in metazoan species, regulates the Hsp70 folding cycle by attenuating Hsp70 ATPase activity and substrate affinity and therefore reducing the rate of refolding measured *in vitro* (Ballinger et al., 1999). In addition to its N-terminus TPR motifs, CHIP also contains a U-box at its C-terminus (Aravind and Koonin, 2001); this RING finger-like domain has ubiquitin ligase activity, and CHIP facilitates the ubiquitylation and subsequent proteasome-dependent degradation of several chaperone substrates (Connell et al., 2001; Jiang et al., 2001; Meacham et al., 2001). Thus, CHIP provides a direct link between the chaperone and proteasome systems, and is postulated to assist in regulating the cellular balance between folding and degradation (McClellan and Frydman, 2001).

Although we now know that CHIP is a negative regulator of Hsp70 refolding activity in vitro and a chaperone-dependent E3 ubiquitin ligase in vivo, the overall endogenous function of CHIP, especially under stress conditions, is unknown. In this study, we set out to examine the effect of CHIP overexpression on the chaperone system and the role of endogenous CHIP during stress response with cells derived from *CHIP* (–/–) mice. Our results provided a mechanism for an unexpected function of CHIP, and thus suggesting a pivotal role of CHIP for the fine tuning of stress response.

2.3 Materials and methods

Cell culture -- COS7 cells were cultured as previously described (Connell et al., 2001). *HSF1* (–/–) and *HSF1* (+/+) murine embryonic fibroblasts were provided by Ivor Benjamin (McMillan et al., 1998; Xiao X, 1999) and *CHIP* (–/–) and *CHIP* (+/+) fibroblasts were cultured according to standard protocols. Recombinant adenoviruses expressing CHIP or control viruses were constructed with the Ad-Easy system and cultures were routinely infected at a multiplicity of infection of five with an infection efficiency of >98%. Western blotting and immunoprecipitations were performed as previously described (Connell et al., 2001).

Reporter gene and electrophoretic mobility gel shift assays (EMSAs) -- Hsp70 promoter:reporter constructs were provided by David Latchman (Stephanou et al., 1999). Reporter constructs were co-transfected in COS7 with a ß-galactosidase reporter (to control for transfection efficiency), with or without plasmids expressing CHIP, CHIP mutants or other co-chaperones as indicated. For EMSA, a radiolabeled HSE oligonucleotide was incubated with nuclear extract from cells treated as indicated. The reaction mixture was incubated at room temperature for 20 min and fractionated on a 5% native polyacrylamide

gel in 0.5x TBE buffer. To determine the specificity of the DNA–protein complexes, we performed competition assays using 50-fold molar excess of the unlabeled double-stranded HSE oligonucleotide (specific inhibitor) or excess of an unrelated NFκB oligonucleotide of comparable length (non-specific inhibitor). To characterize specific DNA-binding proteins, we incubated nuclear extracts with anti-HSF1 or anti-HSF2 antibody before adding probe.

Assays of apoptosis -- *CHIP* (+/+) and *CHIP* (–/–) fibroblasts were incubated at 37°C, or heated at 42°C for 30 min, followed by 6 h recovery at 37°C and heated again at 45°C for 45 min (to stimulate maximal preconditioning), or only heated at 45°C for 45 min. Cells were incubated at 37°C as indicated in the figure legends before lysing. Viability was measured with the XTT Assay (Roche). Caspase 3 activity was measured using a fluorometric assay (Roche). Cleaved caspase 3, which is the activated form of the enzyme, was detected with cleaved caspase 3 (Asp175) antibody 9661 (Cell Signaling Technology), which detects only the active form of caspase 3.

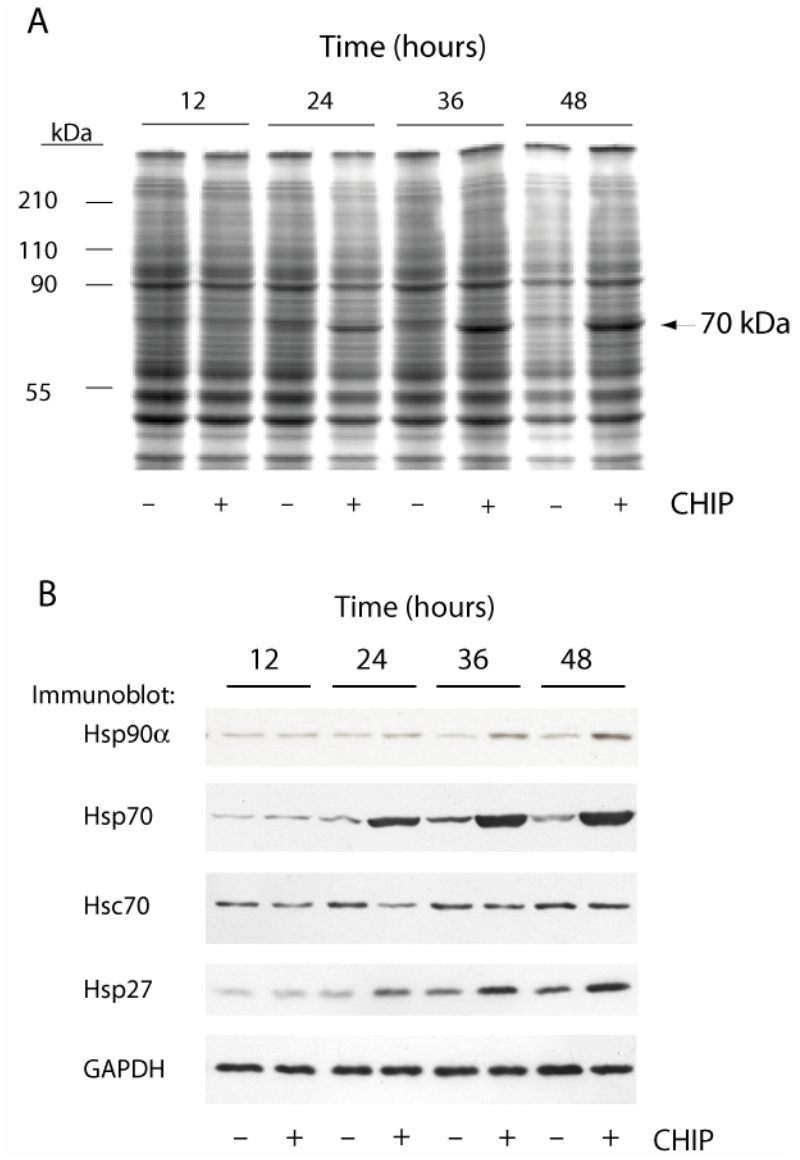


Figure 2.1 Expression of heat shock proteins induced by CHIP overexpression. (**A**) Coomassie Blue gel of lysates from COS7 cells infected with an adenovirus expressing CHIP demonstrates increased expression of a 70-kDa protein compared with cells infected with a control adenovirus. (**B**) Western blot analysis indicates that expression of the chaperones Hsp27, Hsp90α and especially Hsp70 is increased by CHIP.

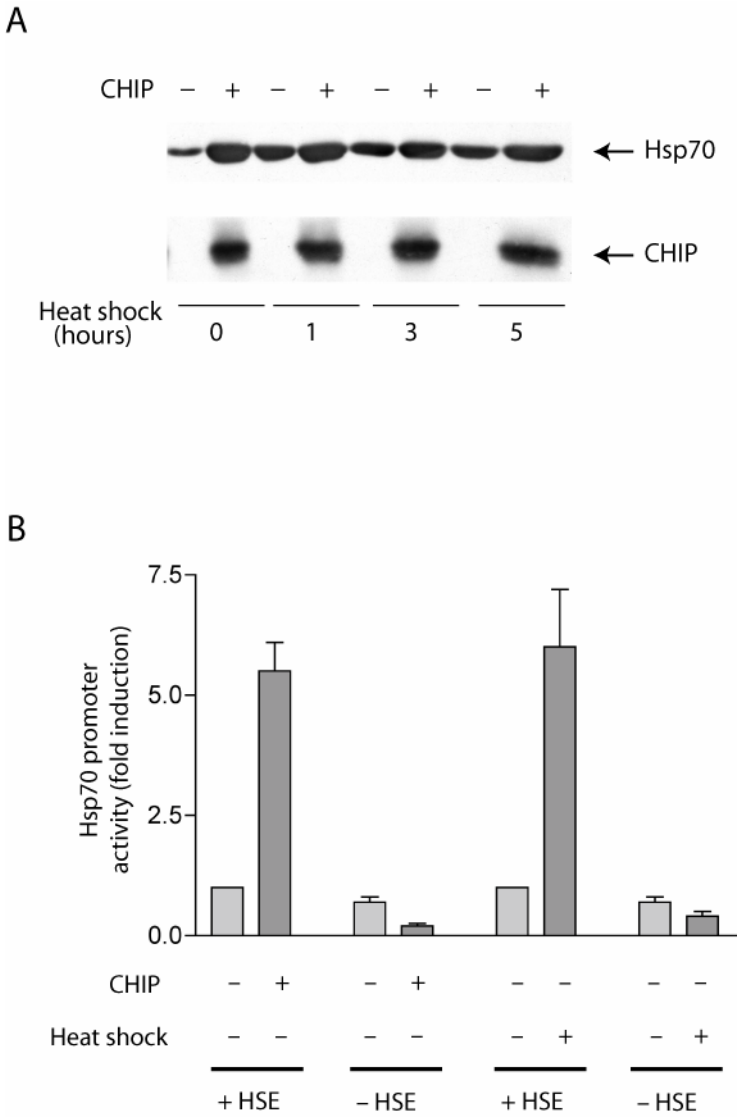


Figure 2.2 Induction of Hsp70 by CHIP is not additive to the effects of heat shock. (**A)** COS7 cells infected for 24 h with an adenovirus expressing CHIP or a control adenovirus were subjected to heat shock at 42°C for the indicated times. Hsp70 induction by CHIP was comparable to, and not additive with, that induced by heat shock. (**B**) Hsp70 promoter:reporter constructs containing (+HSE) or lacking (-HSE) the HSF1 response element were transiently co-transfected with CHIP expression vectors and reporter activity was determined 48 h after transfection, with or without heat shock (42°C). CHIP transactivates the Hsp70 promoter in an HSE-dependent fashion, as does heat shock.

2.4 Results

CHIP increases Hsp70 expression by activation of transcription factor HSF1 -- In order to understand the cellular function of CHIP, we used an adenoviral approach to overexpress CHIP or GFP as a control in COS7 cells, which express CHIP endogenously at low but detectable levels. We examined the protein expression profile of cells at different time point after viral infection with SDS-PAGE followed by Coomassie Blue staining. We found that overexpression of CHIP elevated the levels of a highly inducible 70 kDa protein (Figure 2.1A). Based on the size and abundance of this protein, we suspected that this protein was the inducible form of heat shock protein 70. Western blot analysis confirmed that this protein is indeed Hsp70. In addition, we blotted the membrane with antibodies against several other chaperones, and found that protein levels of Hsp90α and Hsp27 are also increased in cells overexpressing CHIP (Figure 2.1B). Therefore overexpression of CHIP mimics heat shock stimuli in terms of heat shock proteins induction. This unique observation of co-chaperone-dependent Hsp70 expression led us to consider its mechanisms and functional consequences in more detail.

We compared the expression of Hsp70 in cells overexpressing CHIP without heat shock or at different time points after heat shock. We found that induction of Hsp70 was comparable to and not additive with the effects of heat shock (Figure 2.2A). This result suggests that CHIP and heat shock activate Hsp70 through the same pathway. Expression of heat shock proteins by heat shock is controlled at the transcriptional level by the binding of heat shock factor 1 (HSF1) to the heat shock element (HSE) located in the promoters of heat shock proteins (Wu, 1995). We then investigated whether CHIP activated the same pathway as heat shock does. We used reporter gene assays to examine whether heat shock element is

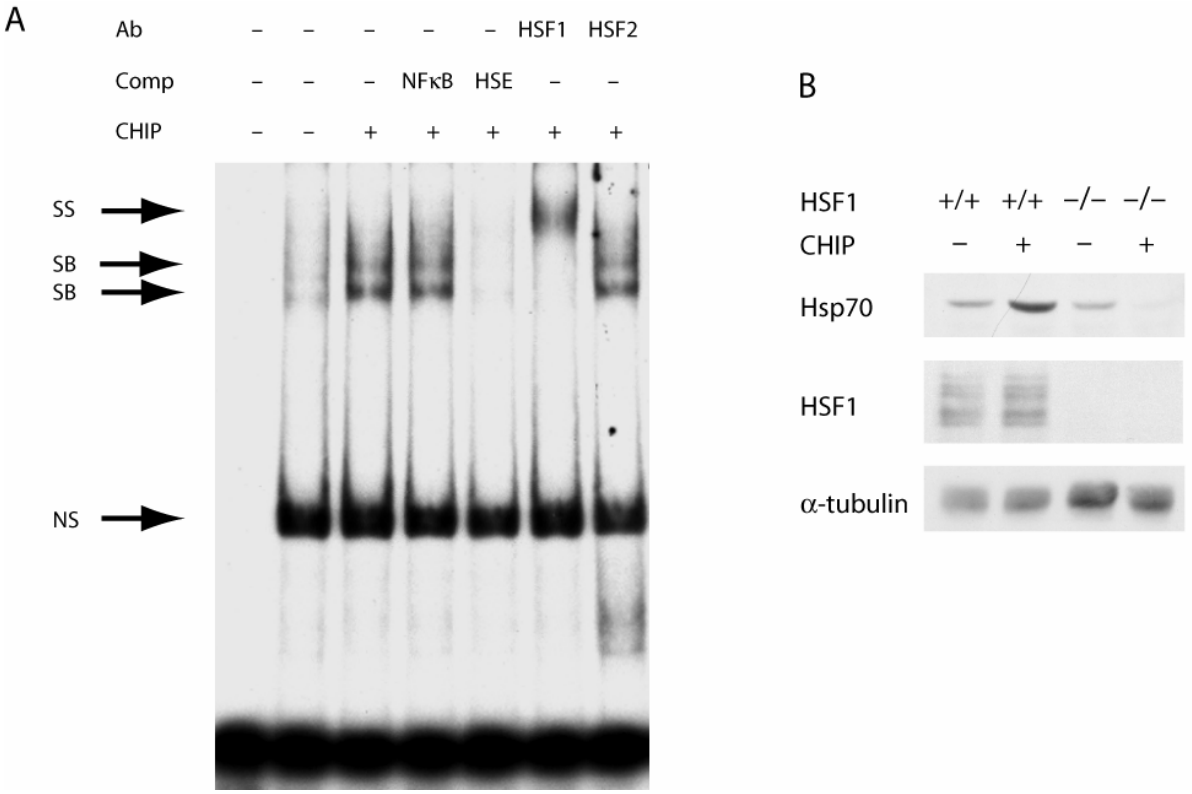


Figure 2.3 Activation of HSF1 by CHIP is required for Hsp70 induction. (**A**) HSF1 DNA binding activity to the HSE was assessed by gel shift assay. Expression of CHIP enhances binding of two specific bands (SB), which are competed away by unlabeled HSE but not by an NFκB consensus sequence. The binding activities can be supershifted (SS) with an HSF1, but not an HSF2, antibody. NS, nonspecific. (**B**) Upregulation of Hsp70 in response to CHIP 48 h after transfection was determined in wild-type or HSF1-null murine fibroblasts by western blot analysis.

required for CHIP-dependent induction of Hsp70. CHIP expression potently activated Hsp70 promoter activity (Figure 2.2B). The activation by CHIP was comparable to that induced by but again not additive with the effect of heat shock and was abolished in a promoter fragment that lacked the HSE, indicating that this effect is dependent on the interaction of HSE with its binding protein(s). We then used gel mobility shift assays to directly examine the protein(s) that mediated the transcriptional effects of CHIP (Figure 2.3A). Overexpressing CHIP enhanced a specific binding of nuclear proteins to the radiolabeled HSE. This binding could be competed by a molar excess of unlabeled HSE but not by a non-specific sequence NF-κB. When we used antibody to HSF1, but not that to HSF2, the mobility of this binding was retarded, proving that HSF1 was the protein that was activated by CHIP. Finally, we confirmed that HSF1 was necessary for the induction of Hsp70 by CHIP in fibroblasts from mice deficient in HSF1 (McMillan et al., 1998; Xiao X, 1999). CHIP upregulated Hsp70 appropriately in wild-type fibroblasts, but not in cells lacking HSF1 (Figure 2.3B). In fact, we observed modest downregulation of basal Hsp70 levels by CHIP in HSF1-null cells. This indicates that CHIP has effects on more than one signaling pathway that regulates Hsp70 expression. HSF1-dependent regulation is clearly the dominant pathway and probably the only positive regulatory pathway affected by CHIP. When this effect is removed, other minor negative regulative pathways become dominant. This also explains the data of figure 2.2B.

It has been well-documented that both Hsp70 and Hsp90 can repress the activation of HSF1 (Morimoto, 1998; Shi et al., 1998; Zou et al., 1998), as can other co-chaperones such as HspBP1 (Hu and Mivechi, 2003), making the activation of HSF1 by CHIP an exceptional observation. To test the specificity of this effect, we examined a panel of co-chaperones for their ability to increase Hsp70 expression and promoter activity. In comparison with other co-

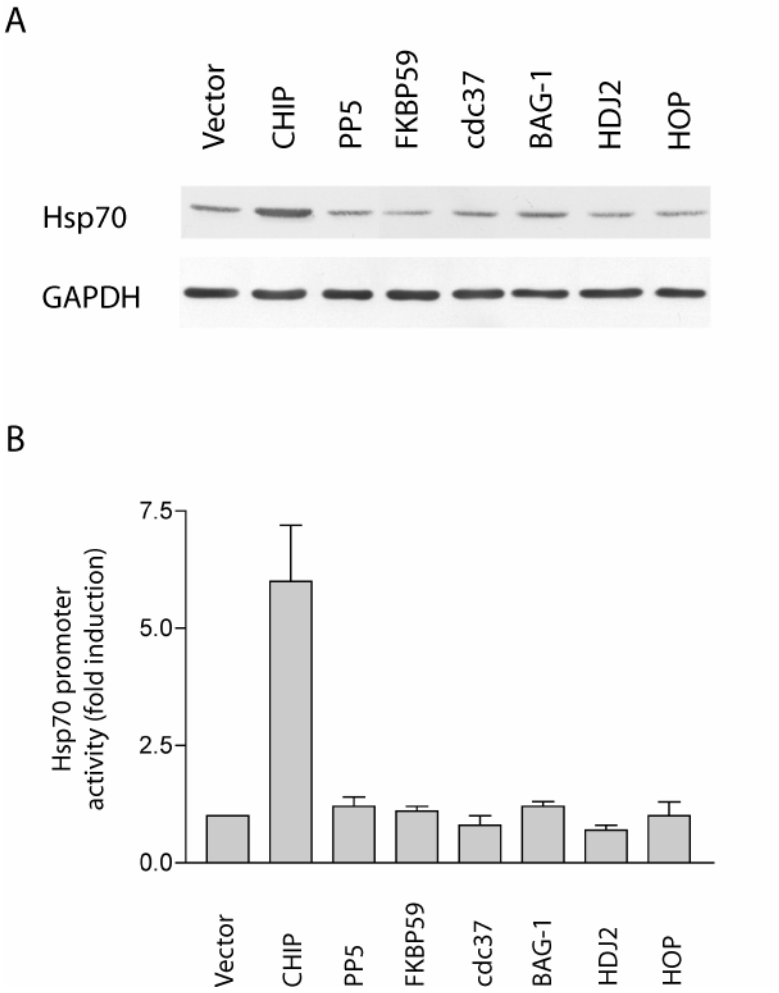


Figure 2.4 Activation of HSF1 is specific to CHIP. (**A** and **B**) Only overexpression of CHIP, but not other co-chaperones [protein phosphatase 5 (*PP5*), FKBP59, cdc37, BAG-1, HDJ2 or HOP], increases Hsp70 protein expression (B) and promoter activity (C) after transient transfection.

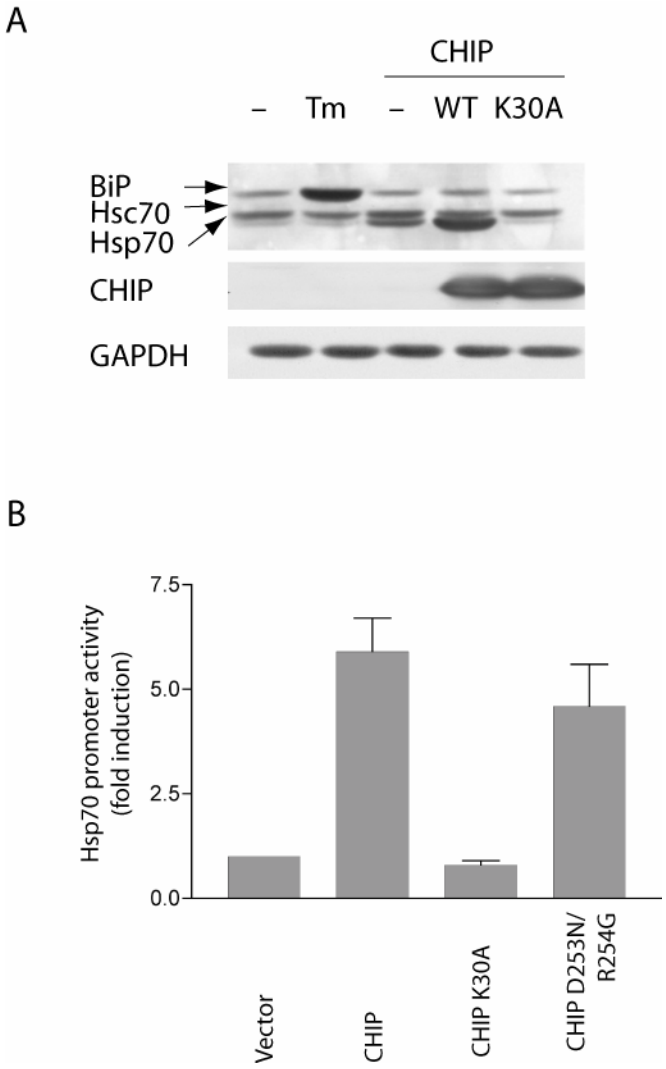


Figure 2.5 Activation of HSF1 is dependent on chaperone interactions. (**A**) Western blot analysis using an antibody that recognizes BiP, Hsc70 and Hsp70 demonstrates that only wild-type CHIP, but not CHIP K30A, increases Hsp70 expression after adenoviral infection, whereas BiP levels are increased by tunicamycin (Tm) but not by CHIP. (**B**) Hsp70 promoter activity is increased by wild-type CHIP and by a mutant lacking ubiquitin ligase activity (CHIP D253N/R254G), but not by a mutant that does not interact with Hsp70/Hsp90 (CHIP K30A).

Chaperones (including protein phosphatase 5, FKBP59 and HOP, each of which contains TPR repeats), only CHIP increased Hsp70 expression (Figure 2.4A), and no co-chaperone other than CHIP significantly activated the Hsp70 promoter (Figure 2.4B).

Several lines of evidence suggest that the induction of Hsp70 by CHIP is not a non-specific stress response generated by increased concentration of misfolded proteins by CHIP overexpression. First, CHIP overexpression neither results in an increased amount of misfolded proteins by metabolic labeling (Meacham et al., 2001), nor inhibits general proteasome function (Jiang et al., 2001). Second, overexpression of CHIP, but not other co-chaperones, induced expression of Hsp70. If we suspect that overexpression of proteins increases the amount of unfolded proteins, it is highly unlikely that only the overexpression of CHIP, but not that of the other co-chaperones, results in the induction of stress response. Third, we performed experiments to test whether overexpression of CHIP activates endoplasmic reticulum stress response. Compared with tunicamycin that activated a potent ER stress response manifested by the induction of BiP, overexpression of CHIP did not affect BiP levels (Figure 2.5A). Importantly, the inducibility of Hsp70 was completely abolished by a mutant form of CHIP (CHIP K30A). This mutant is expressed at similar levels to wild-type protein and differs only in a single amino acid residue in the TPR domain that abolishes interactions with Hsp70 (Scheufler C, 2000); thus, non-specific activation of the stress response by protein overexpression can be excluded. The specificity of this effect raised the question of which features of CHIP contribute to HSF1 activation. To address this, we tested the ability of CHIP mutants to activate the Hsp70 promoter in transient transfection assays. CHIP K30A is unable to activate Hsp70 promoter activity, whereas the activity of CHIP D253N/R254G (which contains mutations in the U-box that abolish ubiquitin ligase activity

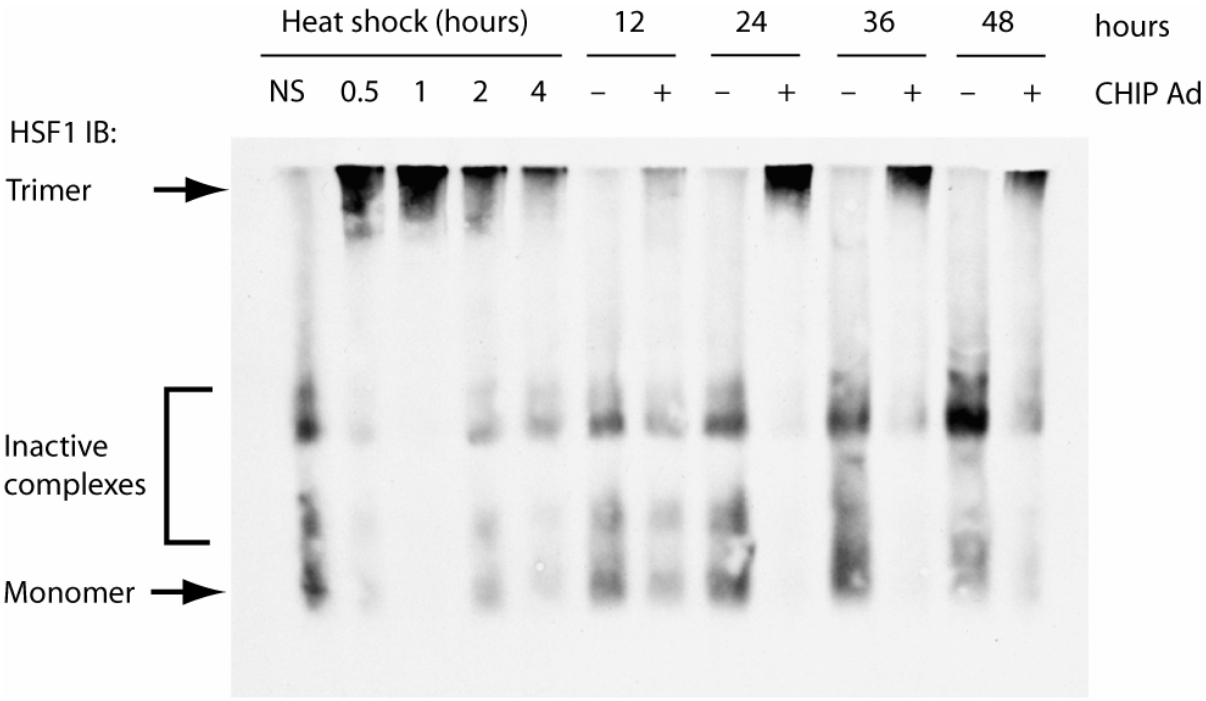


Figure 2.6 Trimerization of HSF1 induced by CHIP overexpression. HSF1 expression was examined by non-denaturing gel electrophoresis in COS7 cells after heat shock (42°C) or after infection with CHIP-expressing adenovirus (or a control adenovirus). Depletion of inactive complexes [which consist of dimers and Hsp70-bound monomers (Liu and Thiele, 1999; Guo *et al.*, 2001)] and quantitative accumulation of the activated trimeric form of HSF1 occurs in CHIP-expressing cells.

(Xu et al., 2002)) was equivalent to that of wild-type CHIP (Figure 2.5B). Thus, the TPR domain of CHIP that is required for the interaction with Hsp70 and Hsp90, but not the U-box that has E3 ligase activity, is required for the induction of Hsp70 by CHIP.

Assembly of a CHIP-dependent activated HSF1 complex – Activation of HSF1 is a multi-step event that includes HSF1 derepression from Hsp70 or Hsp90, trimerization and nuclear localization, binding to HSE and becoming transcriptionally competent. We have shown that activation of HSF1 by CHIP is accompanied by the binding of HSF1 to HSE by the gel shift assay (Figure 2.3A) and transcriptional competency of HSF1 by the reporter gene assays (Figure 2.2B and 2.5B). We then examined whether HSF1 became trimerized by CHIP overexpression. We used a conventional non-denaturing gel separation technique that allows discrimination of monomeric, dimeric, Hsp70-bound and trimeric HSF1 complexes (Zou et al., 1998; Liu and Thiele, 1999; Guo et al., 2001). As a positive control, heat shock induced trimerization of HSF1 peaked at 1 hour after the beginning of heat shock and persisted to 4 hours. CHIP overexpression also activated trimerization of HSF1. However, the kinetics of this trimerization was different from that triggered by heat shock. Trimerization of HSF1 was induced rapidly when overexpressed CHIP was first detectable at 12 hours after transfection, and persisted for at least 48 hours (Figure 2.6, 2.8), suggesting that CHIP-induced trimeric HSF1 complexes may be resistant to attenuation. HSF1 trimers induced by CHIP overexpression had a slightly retarded mobility on PAGE gels, which suggests that posttranslational modification of HSF1 occurred during this process as after heat shock (Wu, 1995). Indeed we observed increased HSF1 phosphorylation by CHIP overexpression in our ^{32}P labeling experiment (data not shown).

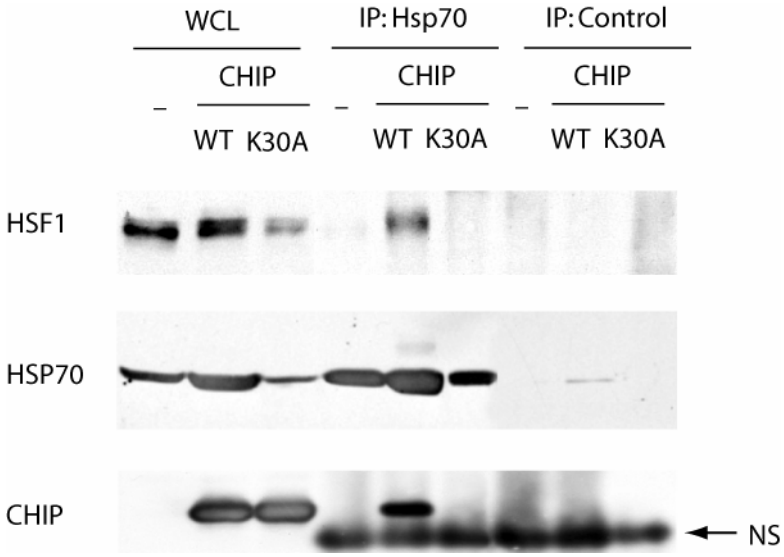


Figure 2.7 Both HSF1 and wild type CHIP, but not the K30A mutant, bind to Hsp70. COS7 cell lysates were prepared 24 h after infection with wild-type (WT) or CHIP or the K30A mutant (or a control adenovirus), and whole cell lysates (WCL) or immunoprecipitates (IP) were probed by western blotting for the presence of HSF1, Hsp70 and CHIP. (NS—non-specific band)

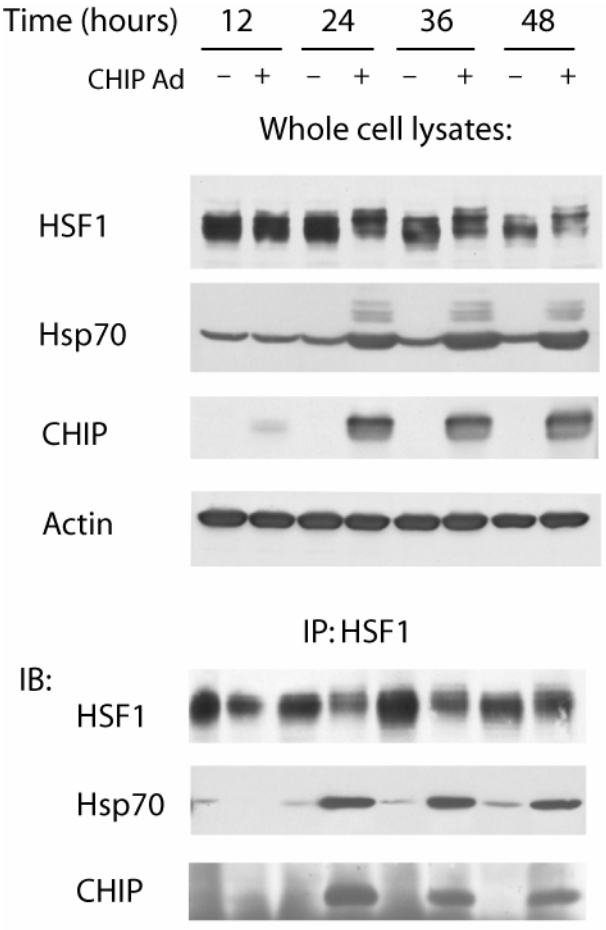


Figure 2.8 HSF1-Hsp70-CHIP form a trimeric complex that is transcriptionally active. COS7 cells were infected with CHIP adenovirus for the indicated times and whole cell lysates or HSF1 immunoprecipitates were probed for HSF1, Hsp70 or CHIP. Hsp70 is stably associated with activated HSF1 in CHIP-expressing cells and CHIP could be precipitated with Hsp70 in these HSF1 immunoprecipitates.

Several studies have demonstrated that both Hsp70 and Hsp90 can repress transcriptional activation of HSF1 (Rabindran et al., 1994; Shi et al., 1998; Zou et al., 1998). Since CHIP interacts with Hsp70/Hsp90, one possible explanation for CHIP-dependent activation of HSF1 is that CHIP disrupts Hsp/HSF1 complexes and thus induces de-repression of HSF1 by Hsp70 or Hsp90. To test this, we examined the effects of CHIP expression on these interactions by co-immunoprecipitation assays. Hsp90/HSF1 interactions remained below the limits of our detection either with or without crosslinkers. However, when we used Hsp70 antibody to precipitate Hsp70 containing complexes, in contrast to the expectation that CHIP would disrupt HSF1/Hsp70 complexes, we detected a weak interaction between Hsp70 and HSF1 in cells expressing GFP control, but a very strong interaction between the two proteins when CHIP is overexpressed (Figure 2.7). These interactions were abolished by mutation of CHIP's TPR domain, suggesting that this HSF1/Hsp70 complex required interactions between CHIP and Hsp70 (Figure 2.7). We also found that when activated HSF1 was co-immunoprecipitated by Hsp70, CHIP was co-immunoprecipitated simultaneously. Since we have not detected direct interaction between CHIP and HSF1, there are two possibilities of these interactions. First, there are two independent complexes, the Hsp70/HSF1 and the Hsp70/CHIP complexes; and second, different domains of Hsp70 establish interactions with HSF1 and CHIP independently and thus these three proteins form a trimeric complex. To test the two possibilities, we performed a converse experiment. We used HSF1 antibody to perform the co-IP experiment. We found that CHIP and Hsp70 were both present in HSF1 immunoprecipitates (Figure 2.8), and the stability of these complexes was similar to that of CHIP-dependent HSF1 trimerization (Figure 2.6). Therefore these results indicate that CHIP,

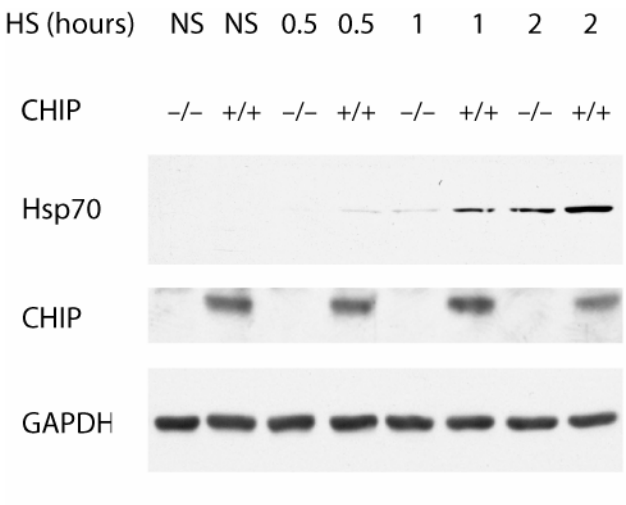


Figure 2.9 Induction of Hsp70 is reduced in *CHIP* (-/-) fibroblasts after heat shock. Western blot analysis of *CHIP* (+/+) or *CHIP* (-/-) fibroblasts after heat shock (HS) at 42°C for the indicated times.

Hsp70 and activated HSF1 exist in an Hsp70-dependent ternary complex that resists HSF1 inactivation.

So far we have shown that CHIP overexpression influences several key steps involved in HSF1 activation. CHIP induces trimerization (Figure 2.6), heat shock protein promoter binding (Figure 2.3A), and transcriptional competency of HSF1 (Figure 2.2D). This activated HSF1 forms a stable trimeric complex with Hsp70 and CHIP that resists attenuation (Figure 2.8), which suggests a mechanism for CHIP dependent HSF1 activation. Activation of heat shock factor and induction of Hsp70 argues for a previously unanticipated role for CHIP in modulating the stress response. Whether this function is significant at the levels of endogenous proteins need to be addressed.

CHIP is required for maximal Hsp70 induction and protection against stress-induced apoptosis -- We isolated dermal fibroblasts from *CHIP* (–/–) mice or wild-type littermates to address the role of endogenous CHIP in regulation of stress response pathways more directly. *CHIP* (–/–) fibroblasts had typical morphologic, growth rate and viability characteristics under standard culture conditions (37°C). However, in comparison with wild-type fibroblasts, *CHIP* (–/–) fibroblasts exhibited markedly diminished responses to stress. We examined Hsp70 induction of these cells after heat shock (42°C) and found that the ability of Hsp70 to be induced was reduced by 50–60% in *CHIP* (–/–) fibroblasts (Figure 2.9A). This finding is consistent with our overexpression studies (Figure 2.1B), and also similar to that which occurs in HSF1-null cells (McMillan et al., 1998). Since Hsp70 induction is crucial during heat shock response to protect cells from injury, we then tested if *CHIP* (–/–) fibroblasts had diminished survival after lethal heat shock (45°C). Indeed, viability of cells measured by XTT metabolism started to decrease in *CHIP* (–/–) fibroblasts

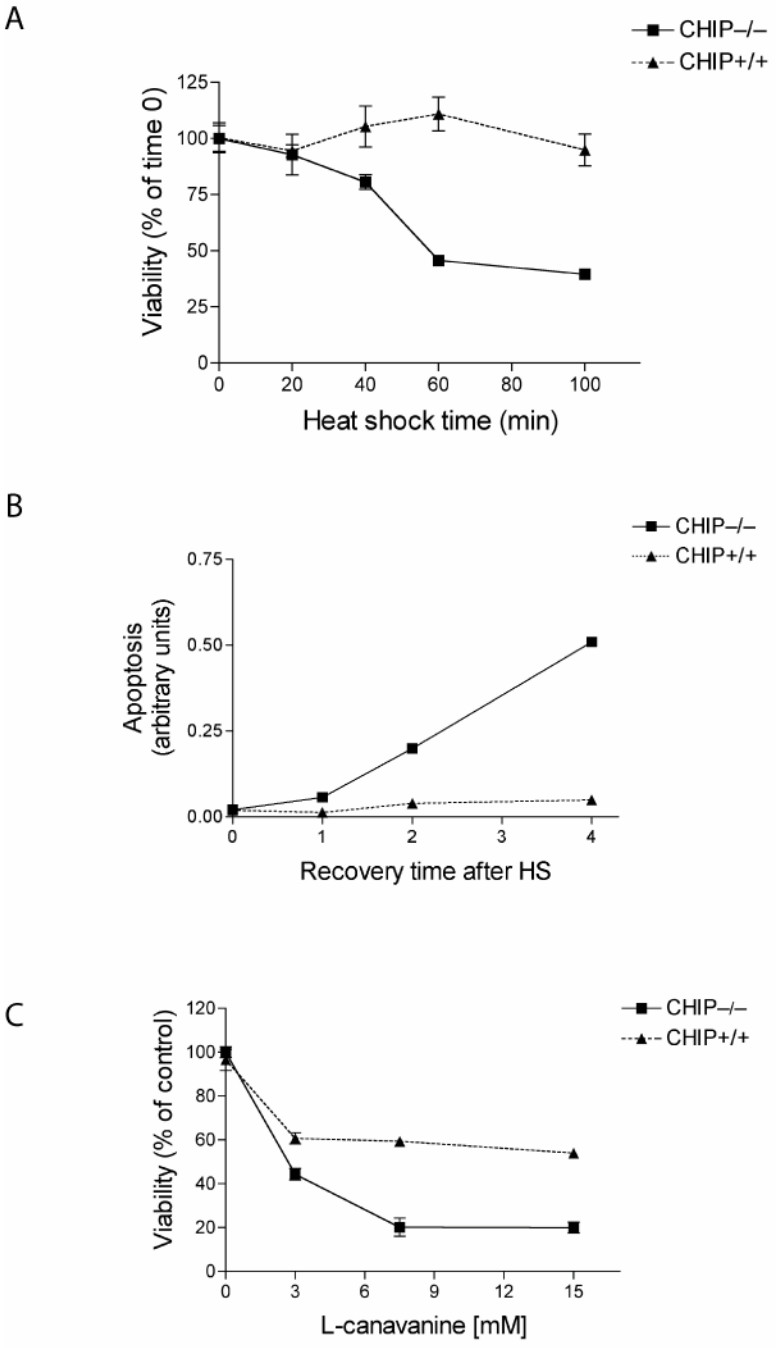


Figure 2.10 Impaired stress response and increased apoptosis in CHIP-deficient fibroblasts. (**A**). The decrease in viability is associated with increased accumulation of oligonucleosomes during recovery from heat shock (45°C for 45 min) for the indicated times as a marker of apoptosis (**B**). (**C**) *CHIP* (-/-) cells are also sensitive to challenge with L-canavanine, an amino acid analog that causes accumulation of misfolded proteins

after 40 minutes of heat shock, with the maximum effect reaching 25% survival after 60 minutes of heat shock; while the survival of *CHIP* (+/+) fibroblasts remained largely unchanged (Figure 2.10A). Since heat stress can activate an apoptotic cascade (Beere and Green, 2001), we then examined whether the reduced survival of *CHIP* (–/–) fibroblasts after heat shock was due to apoptosis. We measured oligonucleosomes formation as an indicator of apoptosis of cells after 60 minutes of heat shock at 45°C, and found that oligonucleosomes began to be detected 1 hour after heat shock in *CHIP* (–/–) fibroblasts, and the amount steadily increased to 4 hours after heat shock; while no oligonucleosomes were detected in *CHIP* (+/+) fibroblasts (Figure 2.10B). The stress-induced defect in *CHIP* (–/–) fibroblasts was not limited to a failure of tolerance to heat shock; cells lacking endogenous CHIP were also more susceptible to L-canavanine, which is an arginine analog that incorporates into proteins during synthesis and induces protein misfolding. Viability of cells treated with L-canavanine decreases in both the *CHIP* (+/+) and *CHIP* (–/–) fibroblasts, but the effect in the *CHIP* (–/–) fibroblasts was more profound (Figure 2.10C). As the primary defect in HSF1 (–/–) fibroblasts is inability to develop thermotolerance after preconditioning with a sublethal heat challenge (42°C) (McMillan et al., 1998), we then examined whether *CHIP* (–/–) cells develop thermotolerance, thus sharing the same pathway that lead to induction of Hsp70. We measured cell viability at 6 hours or 24 hours after lethal heat shock. At the 6 hours recovery time point, viability of *CHIP* (–/–) fibroblasts was lower than that of *CHIP* (+/+) fibroblasts under the same treatments. In *CHIP* (–/–) fibroblasts, preconditioning increased cell viability from 50% to about 75%. Thus, the loss of viability of *CHIP* (–/–) cells after thermal challenge was partially rescued by preconditioning (Figure 2.11A). This suggested to us that CHIP may share a similar mechanism with HSF1 in the development of

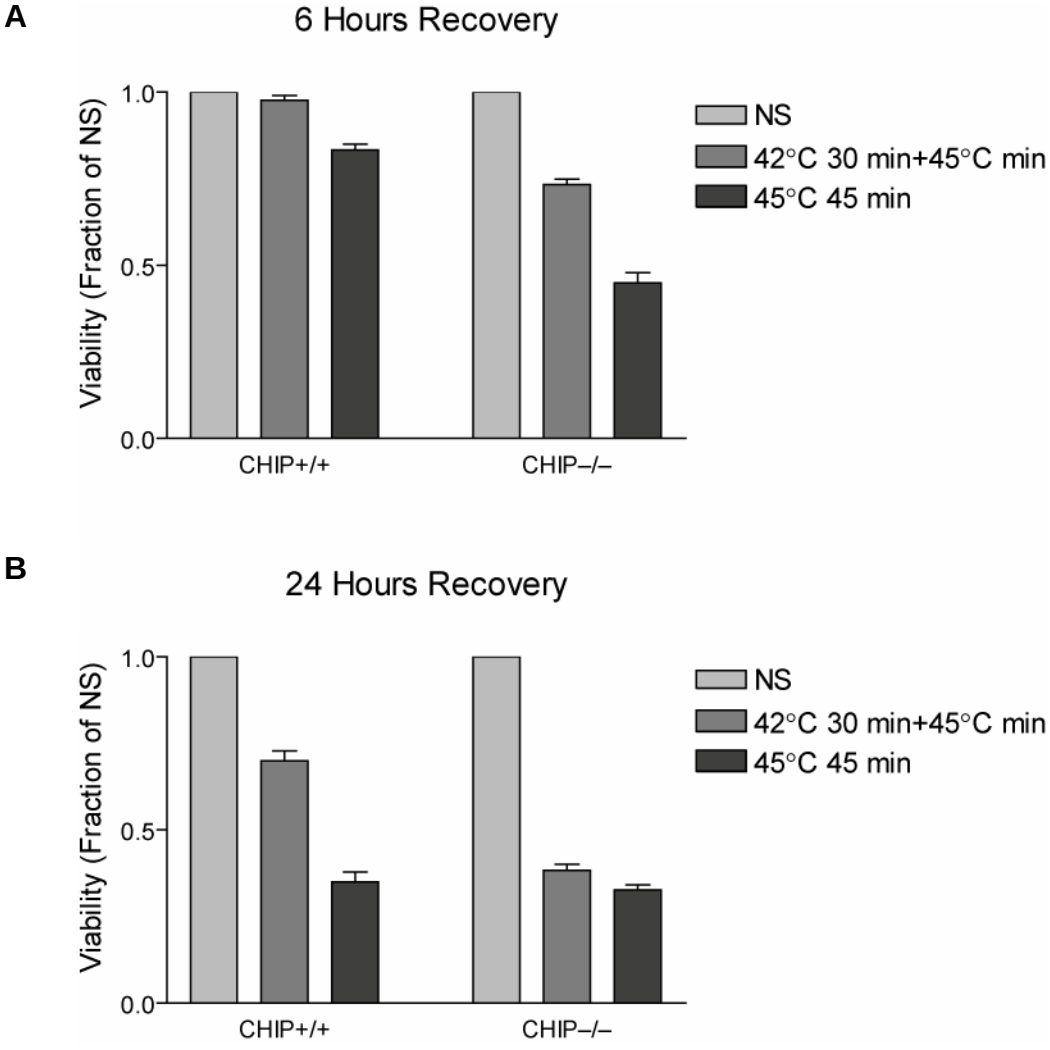


Figure 2.11 Preconditioning partially rescues *CHIP* (-/-) fibroblasts from lethal thermal challenges. (**A** and **B**) *CHIP* (-/-) and *CHIP* (+/+) cells were heat shocked for 45 min at 45°C 6 hours after with or without preconditioning at 42°C for 30 min. Viability of cells was measured after 6 h (A) or 24 h (B) of recovery at 37°C as indicated. *CHIP* (-/-) fibroblasts suffer decrements in viability in the presence or absence of preconditioning. NS, without heat stress.

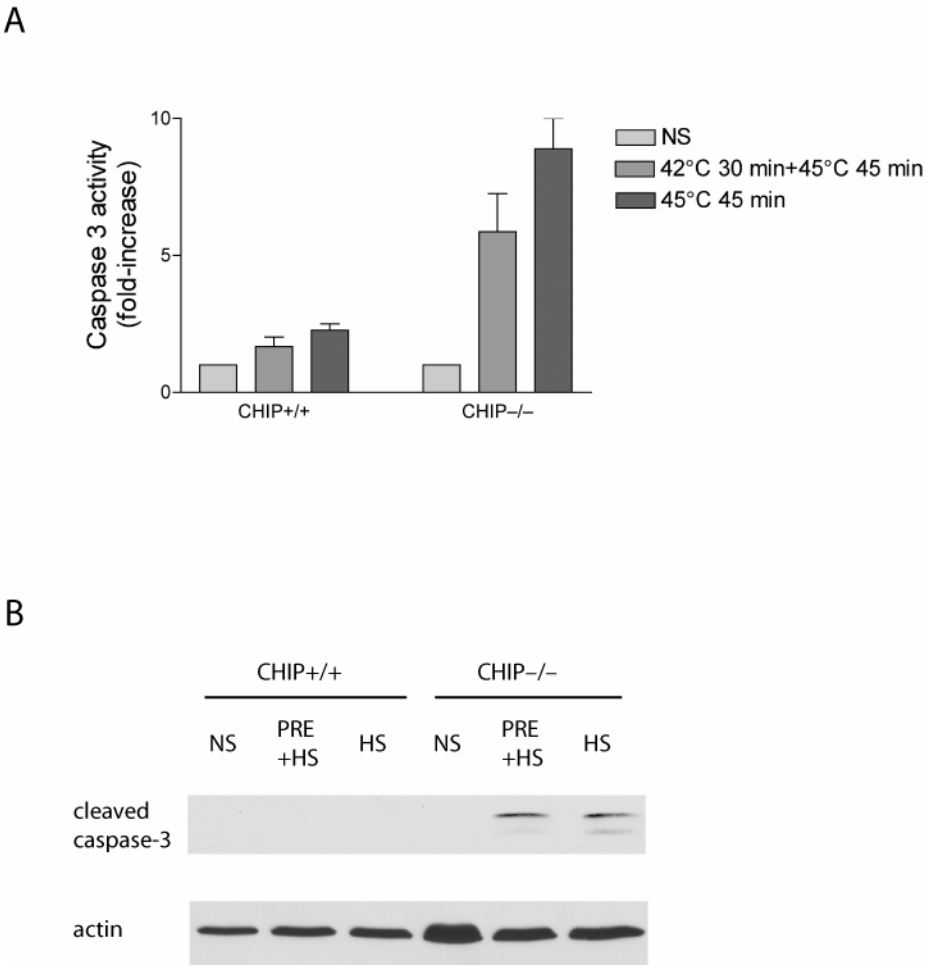


Figure 2.12 Caspase 3 is activated in *CHIP* (-/-) fibroblasts after heat shock. Caspase 3 activity (A) and cleaved caspase 3 levels (B) were measured using a colorimetric assay after thermal challenge (HS) with or without thermal preconditioning (PRE), as indicated.

thermotolerance. We measured caspase 3 activity after thermal stress with or without preconditioning as a biochemical marker of apoptosis. In comparison to wild-type cells, *CHIP* (–/–) cells markedly increased caspase 3 activity following thermal challenge at 45°C, and this increase is only modestly reduced by preconditioning (Figure 2.12A). Similarly, cleaved (and therefore activated) caspase 3 is only detected in *CHIP* (–/–) cells after heat shock, with or without preconditioning (Figure 2.12B). These studies of the functions of endogenous CHIP support our interpretation of CHIP overexpression studies, and indicate that CHIP has an unanticipated central role in regulating the cellular response to stress.

2.5 Discussion

In the present study, we demonstrate that the Hsp70/Hsp90 co-chaperone CHIP is an activator of HSF1 that leads to the induction of heat shock proteins, which is characteristic of heat shock response. Our conclusion is supported by three lines of evidences. First, overexpression of CHIP induces trimerization, HSE binding, transcriptional activation of HSF1, which results in transcription of heat shock proteins 70, 90, and 27. Second, CHIP-Hsp70-HSF1 forms a stable transcriptional active complex on HSE that resists attenuation. Third, in cell lines without expression of endogenous CHIP, there is decreased levels of heat shock response, which is associated with decreased viability and increased apoptosis at times of stress challenges. These results indicate that CHIP is an important regulator of heat shock response.

The prevailing model suggests that Hsp70 and Hsp90 are negative regulators of HSF1. These heat shock proteins are induced during heat shock response and forms a feed back control of HSF1 that leads to repression of HSF1 and attenuation of heat shock response (Morimoto, 1998). However, this model is complicated by the fact that Hsp70 also interacts

with transcriptionally active, HSE bound HSF1 [(Abravaya et al., 1992; Rabindran et al., 1994) and our own observation], and HSF1-bound Hsp70 has little effect on transcriptional activity during the activation phase. Our studies here demonstrate that an additional HSF1–chaperone complex can assemble in the presence of CHIP and that this complex maintains a stably activated form of HSF1 that resists attenuation. Interaction between Hsp70 and HSF1 is mediated by the binding of substrate binding domain of Hsp70 with the transactivation domain of HSF1, which has the feature of substrate-chaperone complex (Shi et al., 1998). Formation of the CHIP-Hsp70-HSF1 trimeric complex requires the TPR domain of CHIP (Figure 2.7), indicating that interaction of the TPR domain of CHIP with the carboxyl terminal EEVD motif of Hsp70 is involved. In case of CHIP-Hsp70-HSF1 trimeric complex, it is plausible that modulation of the ATPase activity or the substrate binding affinity of Hsp70 by CHIP (Ballinger et al., 1999) causes remodeling of the interaction between Hsp70 and HSF1 and subsequent exposure of the transactivation domain of HSF1, leading to sustained transcriptional activation of HSF1. In any event, the activation of HSF1 by CHIP represents another layer of regulation, in addition to that conferred by Hsp70 or Hsp90 alone, for controlling the effects of HSF1, and markedly contrasts with other co-chaperones tested in this context, which universally repress HSF1 (Rabindran et al., 1994; Nair et al., 1996; Shi et al., 1998; Zou et al., 1998; Bharadwaj et al., 1999; Marchler and Wu, 2001). Recently, Daxx, a nuclear protein, has also been suggested to be a positive regulator of HSF1. Daxx cannot initiate the activation of HSF1, but interacts with trimeric HSF1 and is required for its maximum activation (Boellmann et al., 2004). These additional regulations may be necessary to fine-tune the stress response, especially in cells where protein levels or activities of these regulatory factors are under control. Examples of these cells in which CHIP levels are

controlled are metabolically active tissues such as striated muscle (Ballinger et al., 1999) or after proteasome inhibition (Imai et al., 2002).

CHIP was originally identified as an Hsp70 co-chaperone that inhibits the ATPase activity of Hsp70 and thus induces substrate release from Hsp70 and the inhibition of Hsp70 refolding activity in vitro (Ballinger et al., 1999). Subsequently, the U-box of CHIP was found to contain ubiquitin E3 ligase activity and that it ubiquitylates chaperone substrates and sends them to the proteasome for degradation (Hohfeld et al., 2001; McClellan and Frydman, 2001). A global switching of a chaperone substrate off the refolding pathway and a diversion of chaperone substrates to the proteasome for degradation are enormous burdens for the cells to bear, since partially damaged proteins that are otherwise able to regain their native structure and function are degraded, and thus a significant amount of energy that was consumed to synthesize these proteins is wasted. Our finding that CHIP also activates HSF1 and induces the expression of Hsp70, as well as another report that CHIP increases the Hsp70-dependent folding activity within cells (Kampinga et al., 2003), suggests that CHIP increases the total buffering and folding capacity of the cell. The simultaneous enhancement of folding of reversibly damaged proteins and degradation of irreversibly damaged chaperone substrates may account for the cytoprotective effect of CHIP during stress challenges.

Regulation of the apoptotic pathway is part of regulation of the stress response. Molecular chaperones, especially Hsp70, intervene with multiple levels of the apoptotic pathways to exert their anti-apoptotic function (Beere and Green, 2001). In this study, we found that CHIP protected cells from heat shock induced apoptosis. It is conceivable that this anti-apoptotic activity of CHIP is mediated by activation of HSF1 and induction of Hsp70, as well as by enhanced degradation of chaperone substrates. Subsequent studies suggest that

CHIP polyubiquitylates and degrades proapoptotic proteins ASK1 (Apoptosis signal-regulating kinase 1) (Hwang et al., 2005) and p53 (Esser et al., 2005; Hwang et al., 2005). Therefore, the effects of CHIP on both the global stress response and specific apoptotic pathways account for the overall antiapoptotic function of CHIP.

2.6 References

Abravaya K, Myers M, Murphy S and Morimoto R (1992) The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes Dev* 6:1153-1164.

Aravind L and Koonin E (2001) The U box is a modified RING finger - a common domain in ubiquitination. *curr Biol* 10:132-134.

Ballinger CA, Connell P, Wu Y, Hu Z, Thompson LJ, Yin L-Y and Patterson C (1999) Identification of CHIP, a Novel Tetratricopeptide Repeat-Containing Protein That Interacts with Heat Shock Proteins and Negatively Regulates Chaperone Functions. *Mol. Cell. Biol.* 19:4535-4545.

Beere HM and Green DR (2001) Stress management – heat shock protein-70 and the regulation of apoptosis. *Trends Cell Biol.* 11:6-10.

Bercovich B, Stancovski I, Mayer A, Blumenfeld N, Laszlo A, Schwartz AL and Ciechanover A (1997) Ubiquitin-dependent Degradation of Certain Protein Substrates in Vitro Requires the Molecular Chaperone Hsc70. *J. Biol. Chem.* 272:9002-9010.

Bharadwaj S, Ali A and Ovsenek N (1999) Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 In vivo. *Mol Cell Biol* 19:8033-8041.

Boellmann F, Guettouche T, Guo Y, Fenna M, Mnayer L and Voellmy R (2004) DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. *PNAS* 101:4100-4105.

Connell P, Ballinger CA, Jiang J, Wu Y and Patterson C (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol.* 3:93-96.

Esser C, Scheffner M and Hohfeld J (2005) The Chaperone-associated Ubiquitin Ligase CHIP Is Able to Target p53 for Proteasomal Degradation. *J. Biol. Chem.* 280:27443-27448.

Gabai VL, Meriin AB, Mosser DD, Caron AW, Rits S, Shifrin VI and Sherman MY (1997) Hsp70 Prevents Activation of Stress Kinases. A Novel Pathway Of Cellular Thermatolorance. *J. Biol. Chem.* 272:18033-18037.

Guo Y, Guettouche T, Fenna M, Boellmann F, Pratt WB, Toft DO, Smith DF and Voellmy R (2001) Evidence for a Mechanism of Repression of Heat Shock Factor 1 Transcriptional Activity by a Multichaperone Complex. *J. Biol. Chem.* 276:45791-45799.

Hohfeld J, Cyr D and Patterson C (2001) From the cradle to the grave: molecular chaperones that may choose between folding and degradation. *EMBO Rep.* 2:885-890.

Hu Y and Mivechi NF (2003) HSF-1 Interacts with Ral-binding Protein 1 in a Stress-responsive, Multiprotein Complex with HSP90 in Vivo. *J. Biol. Chem.* 278:17299-17306.

Hwang J, Zhang C and Patterson C (2005) C-terminus of heat shock protein 70--interacting protein facilitates degradation of apoptosis signal-regulating kinase 1 and inhibits apoptosis signal-regulating kinase 1--dependent apoptosis. *cell Stress Chaperones* 10:147-156.

Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI and Takahashi R (2002) CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* 10:55-67.

Jiang J, Ballinger CA, Wu Y, Dai Q, Cyr DM, Hohfeld J and Patterson C (2001) CHIP Is a U-box-dependent E3 Ubiquitin Ligase. Identification Of Hsc70 As a Target For Ubiquitylation. *J. Biol. Chem.* 276:42938-42944.

Kampinga HH, Kanon B, Salomons FA, Kabakov AE and Patterson C (2003) Overexpression of the Cochaperone CHIP Enhances Hsp70-Dependent Folding Activity in Mammalian Cells. *Mol. Cell. Biol.* 23:4948-4958.

Liu PC and Thiele DJ (1999) Modulation of human heat shock factor trimerization by the linker domain. *J Biol Chem* 274:17219-17225.

Luders J, Demand J, Schonfelder S, Frien M, Zimmermann R and Hohfeld J (1998) Cofactor-induced modulation of the functional specificity of the molecular chaperone Hsc70. *biol chem.* 379:1217-1226.

Marchler G and Wu C (2001) Modulation of Drosophila heat shock transcription factor activity by the molecular chaperone DROJ1. *Embo J* 20:499-509.

McClellan AJ and Frydman J (2001) Molecular chaperones and the art of recognizing a lost cause. *Nat Cell Biol.* 3:E51-53.

McMillan DR, Xiao X, Shao L, Graves K and Benjamin I (1998) Targeted Disruption of Heat Shock Transcription Factor 1 Abolishes Thermotolerance and Protection against Heat-inducible Apoptosis. *J. Biol. Chem.* 273:7523-7528.

Meacham GC, Patterson C, Zhang W, Younger JM and Cyr DM (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol.* 3:100-105.

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* 12:3788-3796.

Mosser DD, Caron AW, Bourget L, Denis-Larose C and Massie B (1997) Role of the human heat shock protein hsp70 in protection against stress- induced apoptosis. *Mol. Cell. Biol.* 17:5317-5327.

Nair S, Toran E, Rimerman R, Hjermstad S, Smithgall T and Smith D (1996) A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor. *cell Stress Chaperones* 1:237-250.

Rabindran SK, Wisniewski J, Li L, Li GC and Wu C (1994) Interaction between heat shock factor and hsp70 is insufficient to suppress induction of DNA-binding activity in vivo. *mol Cell Biol* 14:6552-6560.

Scheufler C BA, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *cell* 101:199-210.

Schneider C, Sepp-Lorenzino L, Nimmesgern E, Ouerfelli O, Danishefsky S, Rosen N and Hartl FU (1996) Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *PNAS* 93:14536-14541.

Shi Y, Mosser DD and Morimoto RI (1998) Molecularchaperones as HSF1-specific transcriptional repressors. *Genes Dev.* 12:654-666.

Stephanou A, Isenberg DA, Nakajima K and Latchman DS (1999) Signal Transducer and Activator of Transcription-1 and Heat Shock Factor-1 Interact and Activate the Transcription of the Hsp-70 and Hsp-90beta Gene Promoters. *J. Biol. Chem.* 274:1723-1728.

Wu C (1995) Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* 11:441-469.

Xiao X ZX, Davis AA, McMillan DR, Curry BB, Richardson JA, Benjamin IJ. (1999) HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *Embo J* 18:5943-5952.

Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C and Neckers L (2002) Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *PNAS* 99:12847-12852.

Zou J, Guo Y, Guettouche T, Smith D and Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *cell* 94:471-480.

Zuo J, Rungger D and Voellmy R (1995) Multiple layers of regulation of human heat shock transcription factor 1. *Mol. Cell. Biol.* 15:4319-4330.

Chapter 3

Regulation of the cytoplasmic quality control protein degradation pathway by BAG2

Part of this chapter was published as: Dai Q, Qian S-B, Li H-H, McDonough H, Borchers C, Huang D, Takayama S, Younger JM, Ren HY, Cyr DM and Patterson C (2005) Regulation of the cytoplasmic quality control protein degradation pathway by BAG2. *J. Biol. Chem.* 280:38673-38681.

3.1 Abstract

The cytoplasm is protected against the perils of protein misfolding by two mechanisms: molecular chaperones, which facilitate proper folding, and the ubiquitin-proteasome system, which regulates degradation of misfolded proteins. CHIP (carboxyl terminus of Hsp70-interacting protein) is an Hsp70-associated ubiquitin ligase that participates in this process by ubiquitylating misfolded proteins associated with cytoplasmic chaperones. Mechanisms that regulate the activity of CHIP are, at present, poorly understood. Using a proteomics approach, we have identified BAG2, a previously uncharacterized BAG domain-containing protein, as a common component of CHIP holocomplexes in vivo. Binding assays indicate that BAG2 associates with CHIP as part of a ternary complex with Hsc70. In vitro and in vivo ubiquitylation assays indicate that BAG2 is an efficient and specific inhibitor of CHIP-dependent ubiquitin ligase activity. This activity is due, in part, to inhibition of interactions between CHIP and its cognate ubiquitin-conjugating enzyme, UbcH5a, which may in turn be facilitated by ATP-dependent remodeling of the BAG2-Hsc70-CHIP heterocomplex. The association of BAG2 with CHIP provides a cochaperone-dependent regulatory mechanism for preventing unregulated ubiquitylation of misfolded proteins by CHIP.

3.2 Introduction

Cells are the basic building blocks of living organisms and proteins are structural and functional elements of the cell. Integrity of proteins is constantly under challenges of oxidative, chemical and thermal damages. Cells have evolved a sophisticated protein quality control system to maintain protein homeostasis. This system includes the molecular chaperone system that helps to fold or refold partially damaged proteins, and the ubiquitin proteasome system in the eukaryotic cells to degrade irreversibly damaged proteins. Improper protein quality control leads to disturbance of normal cellular functions, and results in a variety of diseases (Wickner et al., 1999).

A "protein triage" model has been proposed that defines partitioning of nonnative proteins between chaperones and the proteasome, although it is not completely clear how decision is made (Wickner et al., 1999). Recently, the co-chaperone/ubiquitin ligase CHIP (carboxyl terminus of Hsp70-interacting proteins) has been implicated in both the protein refolding and degradation pathways. CHIP activates HSF1 (Dai et al., 2003) and increased Hsp70 folding activity of the cells (Kampinga et al., 2003) ; the U-box of CHIP also has E3 ubiquitin ligase activity (Jiang et al., 2001) that ubiquitylates a variety of chaperone-bound cytoplasmic and membrane proteins (Connell et al., 2001; Meacham et al., 2001; Xu et al., 2002). Substrates of CHIP are generally categorized into two groups, which include aggregation-prone proteins that need chaperones during their maturation, such as Hsp70 bound immature CFTR (Meacham et al., 2001), Hsp90 clients ErbB2 (Xu et al., 2002) and glucocorticoid receptor (Connell et al., 2001), and nonnative misfolded proteins that are captured by cytoplasmic chaperones (Murata et al., 2001; Yaguchi et al., 2004). Thus CHIP

promotes both the folding and degradation pathways of the protein quality control system, making CHIP a candidate for regulation of protein triage decision making.

CHIP is constitutively expressed in metabolically active tissues (Ballinger et al., 1999), and protein levels of CHIP are largely unchanged during stress challenges, except one observation of increased CHIP level after proteasome inhibitor treatment of a neuronal cell line (Imai et al., 2002), leaving regulation of CHIP activity more pertinent. CHIP forms homodimers and dimerization is required for its E3 ubiquitin ligase activity by truncation mutation experiments in vitro (Nikolay et al., 2004). Besides that, it is unclear how the activity of CHIP is restrained so that unnecessary ubiquitylation of chaperone substrates is avoided. To gain a better understanding of regulation of CHIP in protein quality control network, we took an inductive approach to identify the components of endogenous CHIP complexes using mass spectrometry. We have found that BAG2 is present in the cytoplasmic chaperone complexes that contain CHIP. BAG2 inhibits CHIP ubiquitin ligase activity by disrupting CHIP-Hsp70 ubiquitin ligase complex and also by interfering with CHIP-E2 ubiquitin conjugase association.

3.3 Materials and methods

Antibodies -- Rabbit polyclonal anti-CHIP and anti-BAG2 antibodies were described previously (Ballinger et al., 1999). Rabbit polyclonal anti-Hsc70 (SPA 816) was from Stressgen. Mouse anti-CHIP monoclonal antibody (colony 67) was produced in collaboration with the UNC Monoclonal Antibody Core Facility. Mouse CFTR R domain-specific antibody (MAB1660) was from R&D systems and the MM13-4 anti-CFTR antibody was from Upstate. Anti-Rat-FITC and anti-mouse Texas Red fluorescent antibodies were from Molecular Probes.

Cell culture and transfection -- To generate stable transfectants, pcDNA3-Myc-CHIP or pcDNA3 alone were transfected into HeLa cells cultured on 100-mm plates. 48 hours after transfection, cells were split and G418 was added at a working concentration of 400 μg/ml. Two weeks after transfection, G418-resistant colonies with isolated with cloning rings and screened for expression by Western blotting. For transient transfections, HEK293 cells were transfected with equivalent concentrations of CFTRΔF508, CHIP and BAG2 plasmids using Effectene (Qiagen) at the DNA:Effectene ratio of 1:5. Cells were lysed with RIPA buffer 24 hours after transfection, and CFTR protein levels were analyzed by SDS-PAGE followed by Western blotting.

Mass spectrometry detection of CHIP associated proteins -- HeLa cells stably expressing Myc-CHIP were cultured in 150-mm plates to confluence. Cells were lysed with RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 1% NP-40 and 1 mM sodium orthovanadate). Anti-Myc agarose (9E10, Santa Cruz Biotechnology) was added to the cell lysates. Agarose beads were then spun down at 500X g for 3 minutes and washed 5 times with RIPA buffer. 2X SDS sample buffer was added to the beads and boiled. After pulse spinning, the supernatants were loaded onto SDS-PAGE. Silver staining was performed using the Invitrogen SilverQuest kit according to standard instructions. Individual gel bands were excised according to protocols described previously (Borchers et al., 2000), and the proteins were in-gel digested and analyzed by combined mass spectrometric approaches. Briefly, the gels were subjected to trypsin proteolysis using a ProGest automated digester (Genomics Solutions). The extracted peptides were analyzed on a MALDI-TOF/TOF (Applied Biosystems) and an ESI Q-TOF (API-US Micromass) equipped with a capillary LC system from Waters. Data were submitted to the

MASCOT database search engine (MatrixScience) for protein identification by peptide mass fingerprinting and sequence tag approaches.

Preparation of expression constructs -- pGEX-4T-1 BAG2 was previously described (Takayama and Reed, 2001). Full length BAG2 was subcloned into pcDNA3-HA for mammalian expression. PCR was performed to generate BAG2 4-90 and 91-211 fragments and cloned into pcDNA3-HA and pGEX-6P-1. His-Hsc70, Myc-CHIP and GST-CHIP plasmids were described previously (Ballinger et al., 1999).

Protein purification -- Recombinant proteins were produced in BL21(DE3)RP cells (Stratagene). The cells were induced with 0.4 mM IPTG at room temperature for 5 hours. The cultures were spun down at 4ºC, dissolved in GST lysis buffer (20mM Tris, pH 8.0, 300 mM NaCl, 2 mM EDTA and 2 mM DTT), sonicated, and cleared by centrifugation. Supernatants were incubated with glutathione-sepharose 4B beads at 4ºC for 2 hours and washed with GST lysis buffer with 0.5% Triton 4 times. In some experiments, the GST tag was cleaved with Precision protease (Amersham Biosciences) for pGEX-6P-1 constructs or thrombin for PGEX-4T-1 constructs. For purification of His-Hsc70, bacterial cultures were lysed in His-lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.05% β-ME, and 1 mM benzamidine), and purified with Ni-NTAagarose beads (Qiagen). Recombinant NBD1-R, CHIP, Hsp70, Hdj2, and UbcH5a were purified as previously described (Younger et al., 2004).

In vitro ubiquitylation reactions -- In vitro ubiquitylation of CFTR NBD1-R domain was performed as described previously (Younger et al., 2004). Briefly, 0.5 μg of purified CFTR-NBD domain was incubated with 4 μM CHIP, 0.4-20 μM BAG1 or BAG2, 2 μM Hsp70, 4 μM Hdj2, 0.3 μg purified rabbit E1 (Calbiochem), 1 mg/ml ubiquitin (Sigma), and

8 μM UbcH4 in 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM MgCl2, 5 mM ATP, 10 mM dithiothreitol for 3 h at 37 °C. Samples were analyzed by SDS-PAGE and blotted with anti-CFTR R domain or anti-Hsp70 antibodies.

Nucleotide binding assays -- Measurement of nucleotide species bound to Hsp70, CHIP, or BAG2 was performed by [α-32P]8-N3ATP photolabeling. 0.5 μg of BSA, 0.3 μg of CHIP, 0.4 μg of BAG2, or 0.5 μg of Hsp70 (to keep equimolar amounts of proteins in each reaction) were incubated with 1 μCi of [α-32P]8-N3ATP (10-15 Ci/mmol) in 20 μl of reaction buffer containing 20 mM Hepes, pH 7.4, 50 mM KCl, and 10 mM MgCl2. After 15 minutes incubation at room temperature, the reactions were irradiated with an ultraviolet transilluminator for 2 minutes. Nucleotides bound to proteins were then separated from the free nucleotides by size exclusion chromatography using a G50 spin column. 1 μl of the flow through were analyzed by liquid scintillation counting.

GST pull-down assays -- Glutathione-sepharose bound proteins were incubated with purified proteins for 2 hours at 4ºC with continuous rotation in GST lysis buffer. The beads were washed 4 times with GST lysis buffer containing 400 mM NaCl. Beads were then boiled in 1X SDS sample buffer and loaded on SDS-PAGE. For His-Hsc70 binding assays, binding reactions were performed in His-lysis buffer with purified BAG2 and CHIP. When indicated, some experiments were performed in the absence or presence of ATP, ADP, or the non-hydrolyzable ATP analogAMP-PNP (5 mM)**.**

3.4 Results

Identification of CHIP-associated proteins by mass spectrometry – In order to analyze the endogenous CHIP-containing protein complexes, we created a HeLa cell line

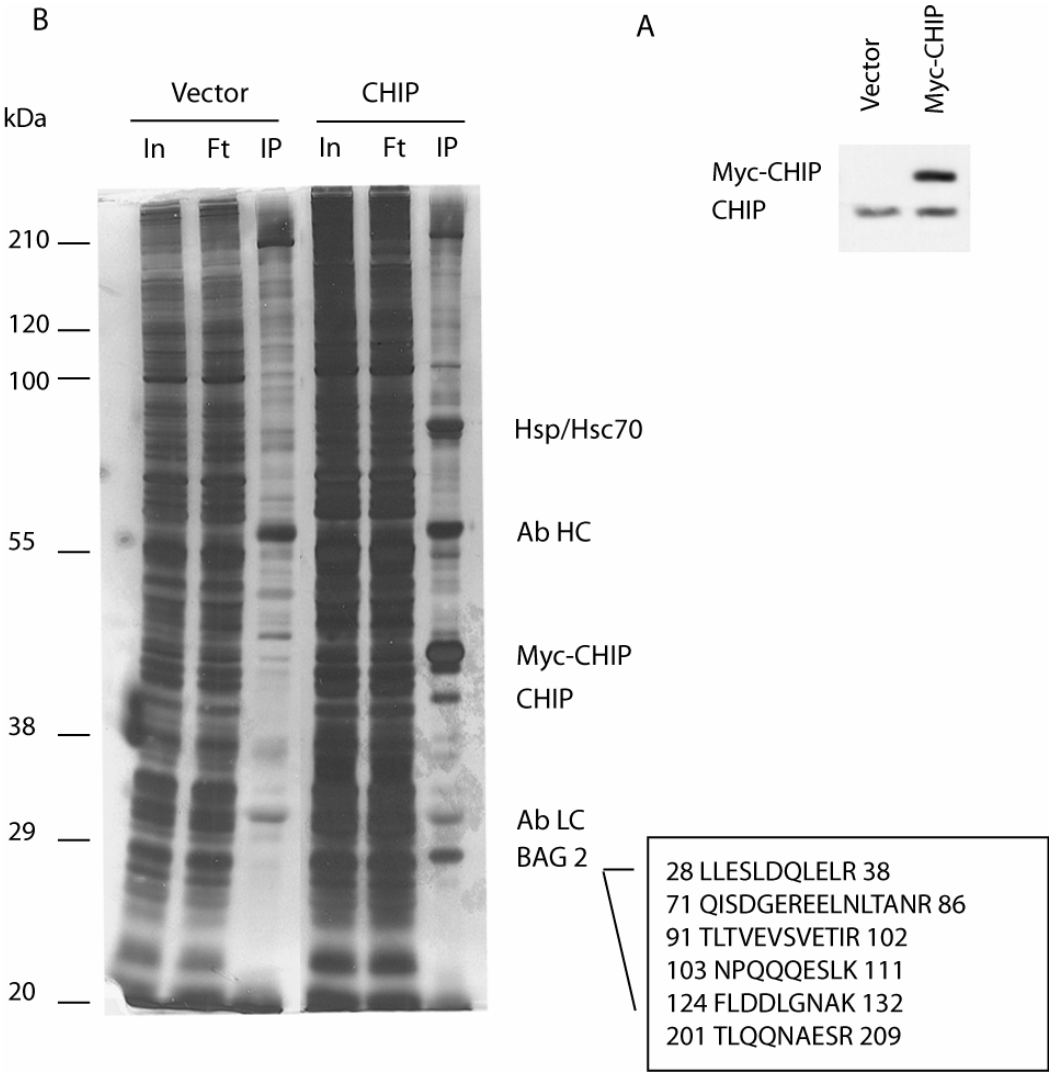


Figure 3.1 Mass spectrometry identification of BAG2 as a component of CHIP-containing complexes in vivo. *A.* HeLa cell lines were stably transfected with a plasmid containing Myc-tagged CHIP or vector alone. Clones expressing Myc-CHIP at near-endogenous levels, as confirmed by Western blot analysis, were selected and used to identify endogenous proteins present in CHIP complexes in vivo. *B.* Silver staining of input (In), flowthrough (Ft), and Myc immunoprecipitates (IP) from these HeLa cell lines indicates the proteins that co-purify with CHIP. A 26-kDa protein was sequenced by mass spectrometry, and the 6 indicated peptide sequences were identified which led to the conclusion that this protein was BAG2.

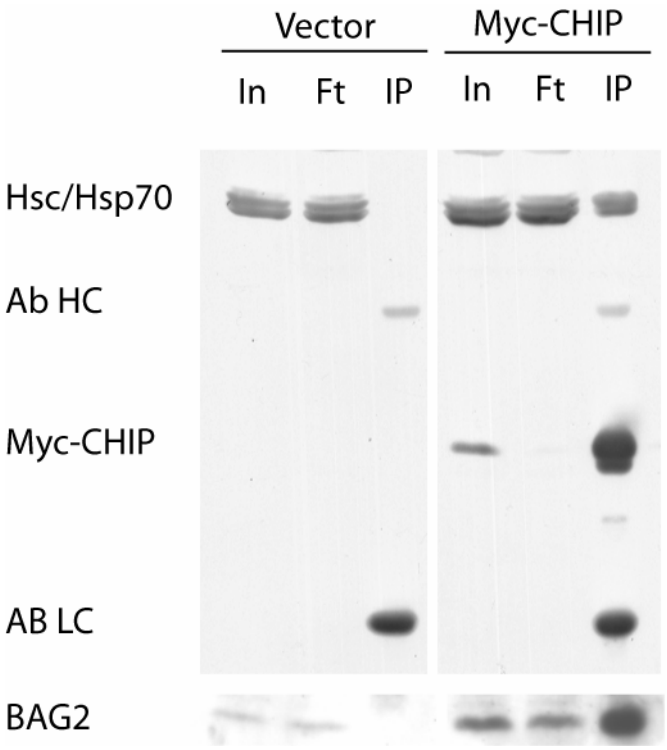


Figure 3.2 Western blotting confirmed BAG2 as a component of CHIP-containing complexes. Western blot analysis with antibodies against Hsp/Hsc70, CHIP, and BAG2 confirmed the identities of these proteins in immunoprecipitates from Myc-CHIP-expressing cells.

that stably expresses Myc-tagged CHIP at levels approximating endogenous expression, and a cell line that is transfected with the parent vector alone (Figure 3.1*A*). Low-level expression of the transgene avoided CHIP-dependent activation of heat shock factor-1 (Dai et al., 2003), and also enabled us to detect the complexes that were similar to the endogenous CHIP-containing complexes. Lysates from these cell lines were probed with an anti-Myc monoclonal antibody, and immunocomplexes were resolved by electrophoresis followed by silver staining to detect individual components. Representative immunocomplexes are shown in Figure 3.1*B*. Individual bands were excised and analyzed by MALDI TOF/TOF and ESI Q-TOF (Borchers et al., 2000). Consistent with previous observations, peptides representing Hsc/Hsp70 and CHIP were identified in bands of the appropriate molecular weights, indicating that CHIP associates with chaperones and homodimerizes (Ballinger et al., 1999; Nikolay et al., 2004). In addition, a 28-kDa band that was present in approximate stoichiometry with Hsc70 in CHIP immunocomplexes was identified. 6/6 resolvable spectra coded fragments of BAG2. We further confirmed the identity of this band by using an anti-BAG2 antibody and we found this protein was enriched in CHIP-containing immunocomplexes (Figure 3.2).

BAG2 is one of six proteins in mammalian cells that contain the BAG domain. BAG1, the founding member of this family, was originally identified as a Bcl-2-binding protein. Subsequently it was confirmed to associate with Hsc70 as a co-chaperone (Takayama S, 1995; Takayama et al., 1997). All the BAG domains of the BAG family proteins are located in the C-terminus of these proteins, and are able to interact with the ATPase domain of Hsp70 (Takayama et al., 1999). This interaction induces a conformational change of the ATPase domain, which results in nucleotide exchange (Sondermann et al., 2001). In addition

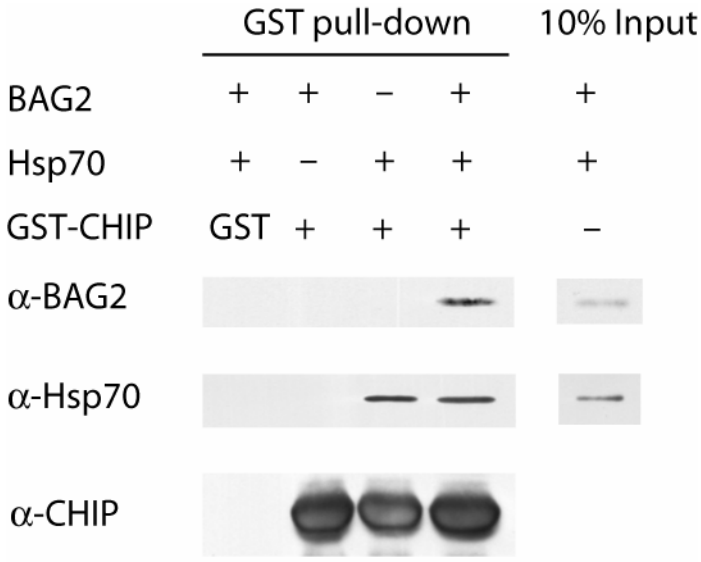


Figure 3.3 GST-CHIP pulls down BAG2 via Hsp70 in vitro. In vitro pull-down assays with GST-CHIP (or GST alone) were used to test the associations of CHIP with BAG2 in the presence or absence of Hsp70. In these assays, CHIP efficiently interacted with BAG2, but only when Hsp70 was present in the binding assays.

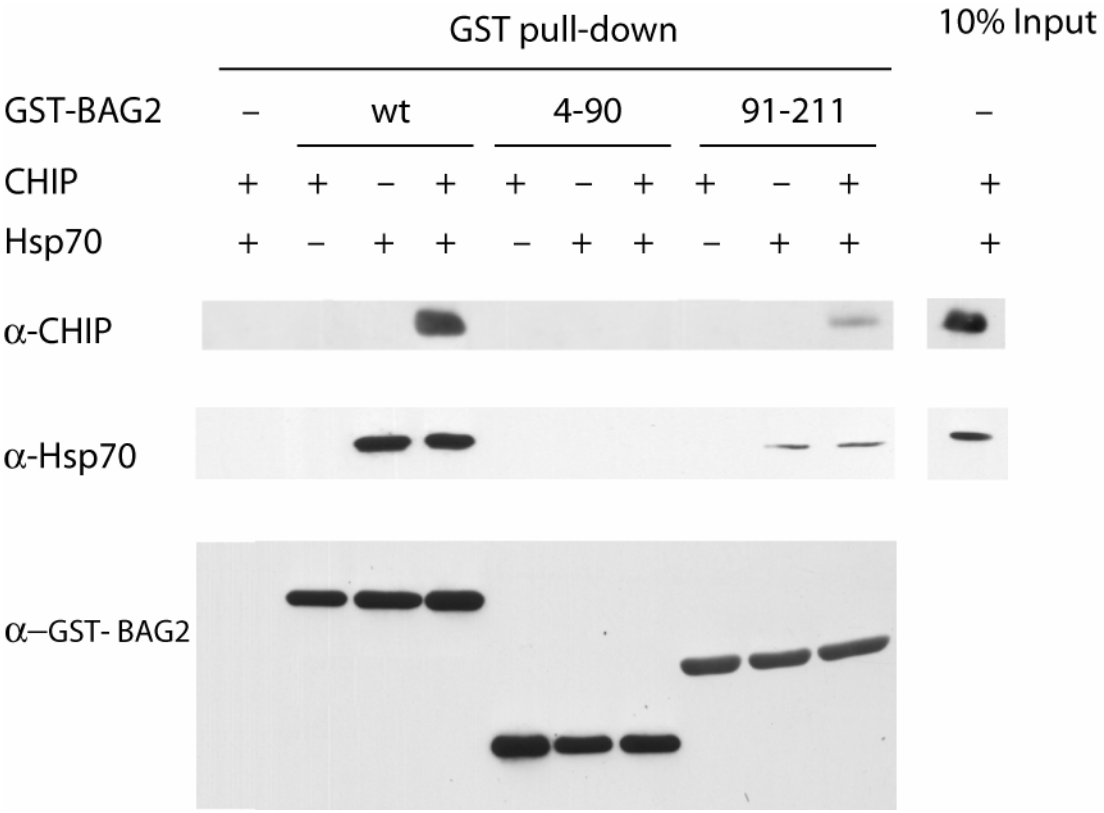


Figure 3.4 BAG domain of GST-BAG2 pulls down CHIP via Hsp70 in vitro. In vitro binding of recombinant CHIP and Hsp70 with full-length GST-BAG2 or BAG2 fragments containing amino acids 4-90 or 91-211 (BAG domain) was tested in GST pull-down assays. In these assays, BAG domain of BAG2 pulls down CHIP with a much less efficiency compared with full-length BAG2 in the presence of Hsp70.

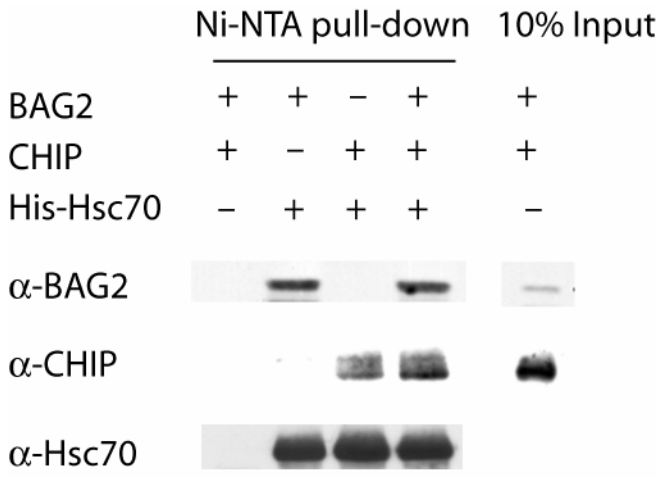


Figure 3.5 His-Hsc70 pulls down CHIP and BAG2 without competition in vitro. Associations of proteins with Hsc70 (tagged with His), with or without BAG2 and/or CHIP, were tested in binding assays using immobilized nickel. In these assays, both CHIP and BAG2 associated with Hsc70 independently, and their association with Hsc70 was not competitive when both CHIP and BAG2 were present.

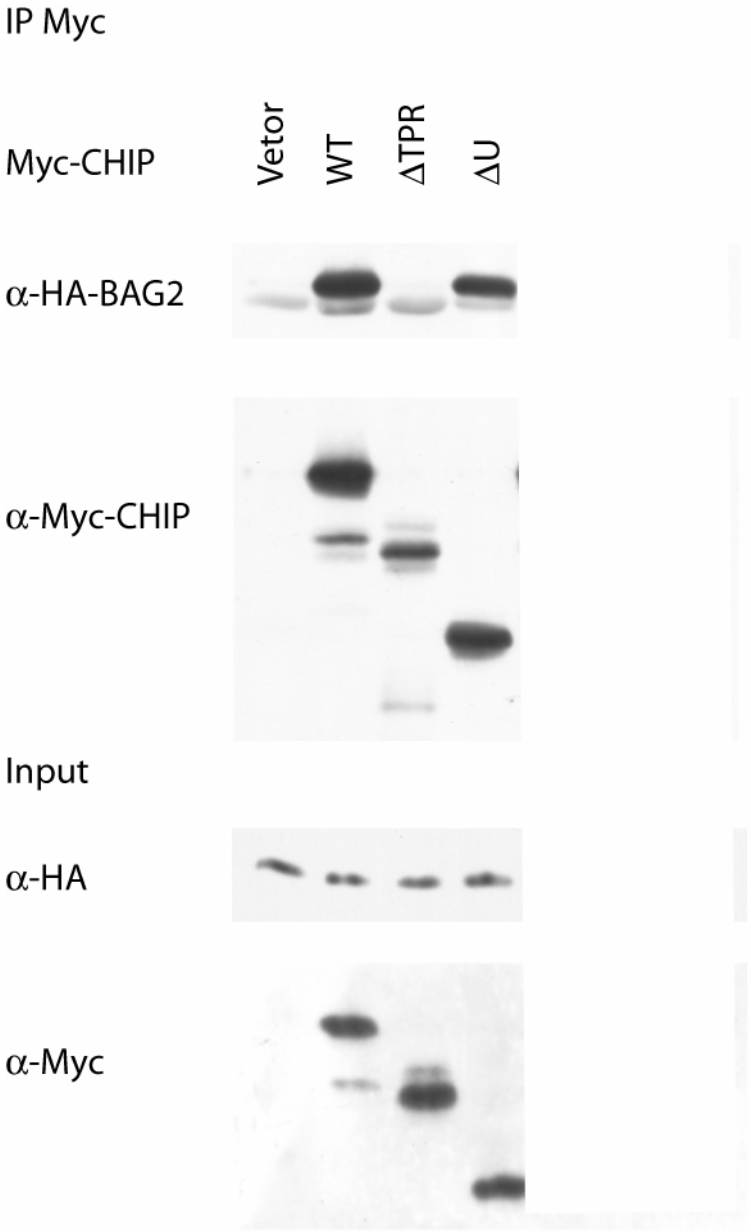


Figure 3.6 Interaction of CHIP with BAG2 requires the TPR domain of CHIP in HeLa cells. HeLa cells were co-transfected with HA-BAG2 and different truncation mutants of Myc-CHIP. Co-immunoprecipitation were performed using anti-Myc antibody and Western blotting were used to detected CHIP and BAG2 in these immunocomplexes. Wild type CHIP and CHIPΔU box pulled down HA-BAG2 with equal affinity, while CHIPΔTPR could not pull down HA-BAG2.

to the BAG domains, BAG-family proteins also contain a diversity of domains, which allow them to interact with specific target proteins or which target them to specific locations within cells (Takayama and Reed, 2001). BAG2 contains the most divergent BAG domain among this family at its carboxyl terminus, and the 90 amino-proximal residues contain no motifs except for coiled-coil structure recognized by the SMART and PFAM algorithms. No cellular functions have been assigned to this protein to date. Interestingly, BAG1 has been implicated in CHIP-dependent protein degradation (Demand et al., 2001), although we did not discover BAG1 in these experiments nor in other tests for endogenous CHIP interaction partners (*data not shown*).

Next, we used in vitro binding assays with purified proteins to verify and characterize the observed interaction between CHIP and BAG2. When we used GST-CHIP, we could only pull-down BAG2 in the presence of Hsp70 (Figure 3.3). In a converse experiment, GST-BAG2 precipitated CHIP in an Hsp70-dependent fashion (Figure 3.4). Deletions of either the BAG domain (amino acids 91-211) or the amino terminal extension (amino acids 4-90) revealed that the BAG domain recruited both Hsp70 and CHIP, but with markedly lower efficiency than native BAG2, suggesting an additional role for the BAG2 amino terminus in efficient complex assembly. Finally, we used nickel chromatography to isolate recombinant proteins associated with His-tagged Hsc70. Both CHIP and BAG2 bound Hsc70 efficiently and without competition (Figure 3.5). For reasons discussed below, it is important to note that the preceding experiments were done in the absence of ATP. To examine domains of CHIP required in the complex formation, we transfected HeLa cells with HA-BAG2 and different truncation mutants of Myc-CHIP. We found that HA-BAG2 could be efficiently pulled-down by wild type and U-box deletion mutant, but not the TPR domain

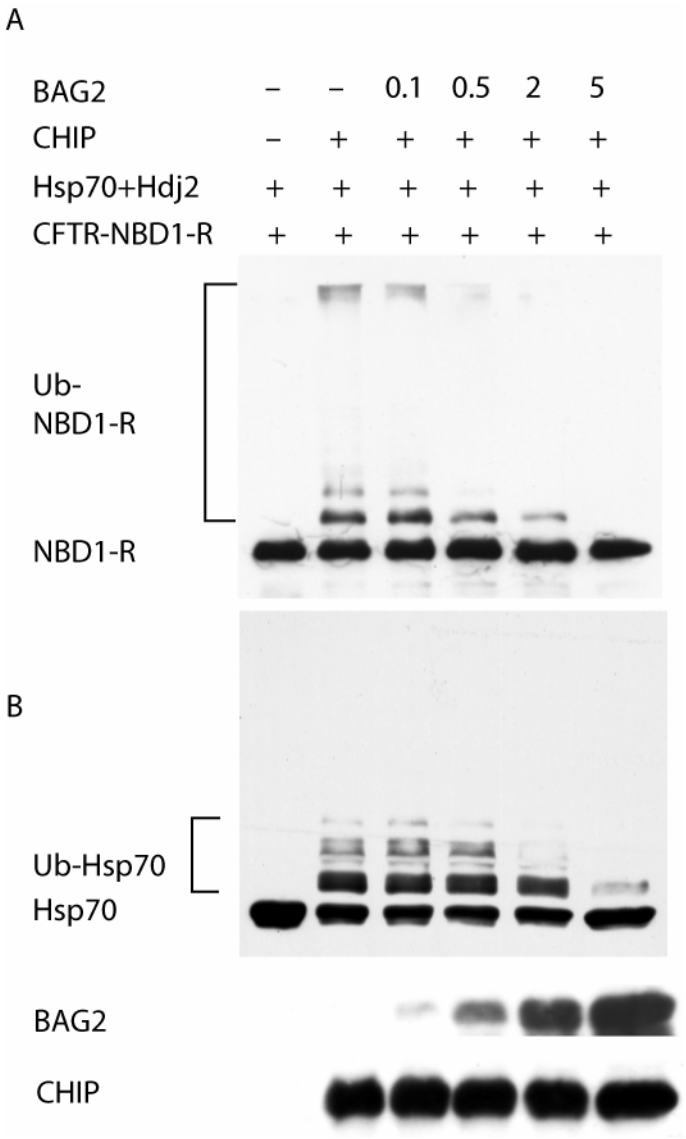


Figure 3.7 Inhibition of CHIP ubiquitin ligase activity by BAG2. *A.* In vitro ubiquitylation assays were performed in reactions containing E1, UbcH5a, CHIP, Hsp70, HDJ2, and the indicated molar ratios of BAG2 to CHIP. The NBD1-R domain of CFTR served as a substrate in these reactions. Western blotting for NBD1 was used to identify its ubiquitylated forms. *B.* Similar in vitro reactions were performed with Hsp70 as the substrate.

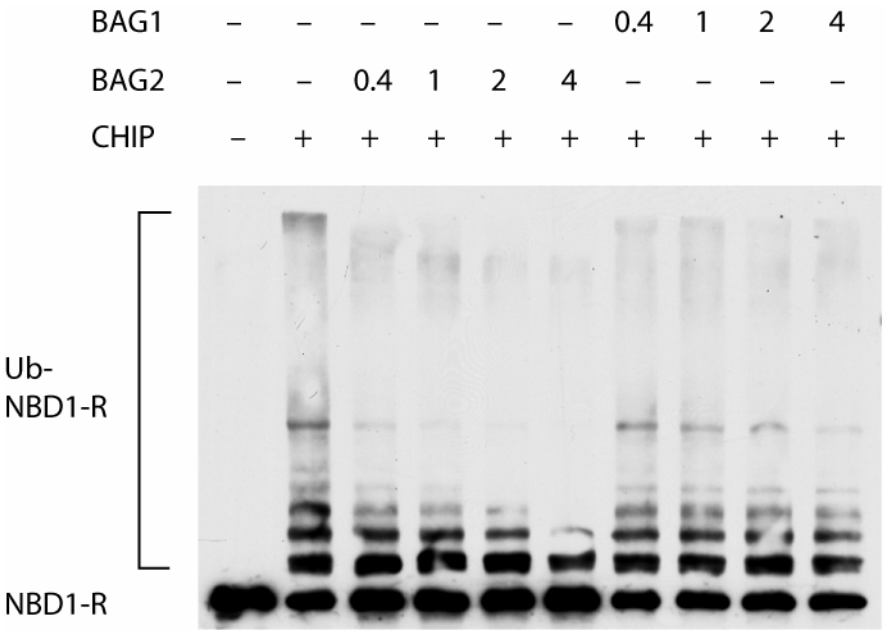


Figure 3.8 Comparison of BAG2 with BAG1 in their ability to inhibit CHIP ubiquitin ligase activity. The antiubiquitylation activities of BAG1 and BAG2 were compared in in vitro ubiquitylation assays using CFTR NBD1-R as a substrate. Ten-fold higher concentration of BAG1 compared with BAG2 was required to achieve similar inhibitory effects.

deletion mutant (Figure 3.6) , which cannot associate with Hsc/Hsp70 (Ballinger et al., 1999). These results, together with previous observations (Takayama and Reed, 2001), suggest Hsp70 acts as a bridge that brings BAG2 and CHIP into a trimeric protein complex, with its ATPase domain interacting with BAG domain of BAG2 and its carboxyl terminal EEVD motif interacting with TPR domain of CHIP, respectively. This complex appears to be further stabilized by interactions involving the amino terminus of BAG2 binding to other sites within this complex; these interactions were explored further in subsequent studies (*see below*).

BAG2 inhibits the ubiquitin ligase activity of CHIP -- CHIP regulates both arms of the cytoplasmic quality control: CHIP directly activates heat shock factor-1 (which transcriptionally regulates molecular chaperones) (Dai et al., 2003) and increases the folding capacity of the cells (Kampinga et al., 2003), and CHIP is a chaperone-dependent ubiquitin ligase (Jiang et al., 2001) that selectively degrades chaperone substrates. Using a variety of assays, we did not identify any consistent effects of BAG2 on CHIP dependent heat shock factor-1 activation (*data not shown*). However, when we used a well-defined CHIP substrate—NBD1 domain of CFTR—to test the ubiquitin ligase activity of CHIP, we found that activity of CHIP was efficiently inhibited by BAG2 (Figure 3.7). Half-maximal effects of BAG2 occurred at roughly stoichiometric concentrations of BAG2 relative to CHIP. We detected ubiquitylation of Hsp70 since it is also a substrate of CHIP (Jiang et al., 2001), and found that addition of ubiquitin residues to this protein is inhibited in a degree similar to that of NBD1 (Figure 3.7*B*).

To distinguish whether the inhibition of CHIP E3 ligase activity is specific to BAG2 or general to all BAG domain containing proteins, and to compare the activity of BAG2 with

BAG1 since BAG1 has been placed in complexes with CHIP in in vitro studies (Demand et al., 2001), we performed in vitro NBD1 ubiquitylation assays with increasing dose of BAG2 or BAG1. Even at 10-fold higher molar ratio compared with BAG2, BAG1 had negligible effects on CHIP ubiquitin ligase activity (Figure 3.8). These results are consistent with previous finding that BAG1 does not affect E3 ligase activity of CHIP (Demand et al., 2001), and indicate that the presence of a BAG domain alone is insufficient to inhibit CHIP activity. In addition, these experiments confirm the specificity of BAG2 as an inhibitor of CHIP ubiquitin ligase activity in this assay.

BAG2-dependent remodeling of the CHIP ubiquitin ligase complex – To explore the mechanism of BAG2-dependent inhibition of CHIP ubiquitin ligase activity, we considered several possible regulatory points that can be utilized by BAG2, based on our current understanding of CHIP activity. First, CHIP forms homodimers and dimerization is required for its ubiquitin ligase activity (Nikolay et al., 2004). Therefore, manipulation of dimeric states of CHIP is a possible candidate. Second, CHIP-Hsp70 forms an E3 ubiquitin ligase complex so that U-box of CHIP brings ubiquitin charged E2 ubiquitin conjugase, and the substrate binding domain of Hsp70 brings ubiquitylation substrates. Since BAG2 is a nucleotide exchange factor for Hsp70 that induces substrate release, it could be the mechanism of BAG2 dependent inhibition. However, CHIP dependent ubiquitylation of Hsp70 is inhibited by BAG2, making it less likely that release of Hsp70 substrates accounts for the inhibition of E3 ligase activity by BAG2. It is also possible that BAG2 disrupts Hsp70-CHIP or CHIP-E2 interaction.

To test whether BAG2 disrupted CHIP homodimers, we co-expressed both Myc and FLAG-tagged CHIP in cultured cells and used the different tags to assay CHIP dimerization

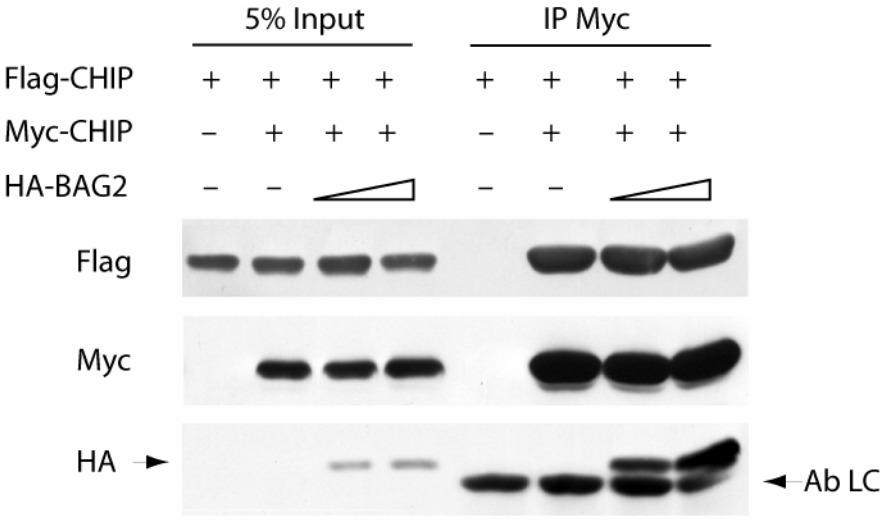


Figure 3.9 BAG2 does not disrupt CHIP dimers. Vectors expressing Flag- and Myc-tagged CHIP (1 μg each) were co-transfected in HeLa cells along with increasing concentrations of a plasmid expressing HA-tagged BAG2 (1-5 μg). Cell lysates were immunoprecipitated with an anti-Myc antibody and then blotted to detect Flag-CHIP and HA-BAG2 that associated with Myc-CHIP in vivo.

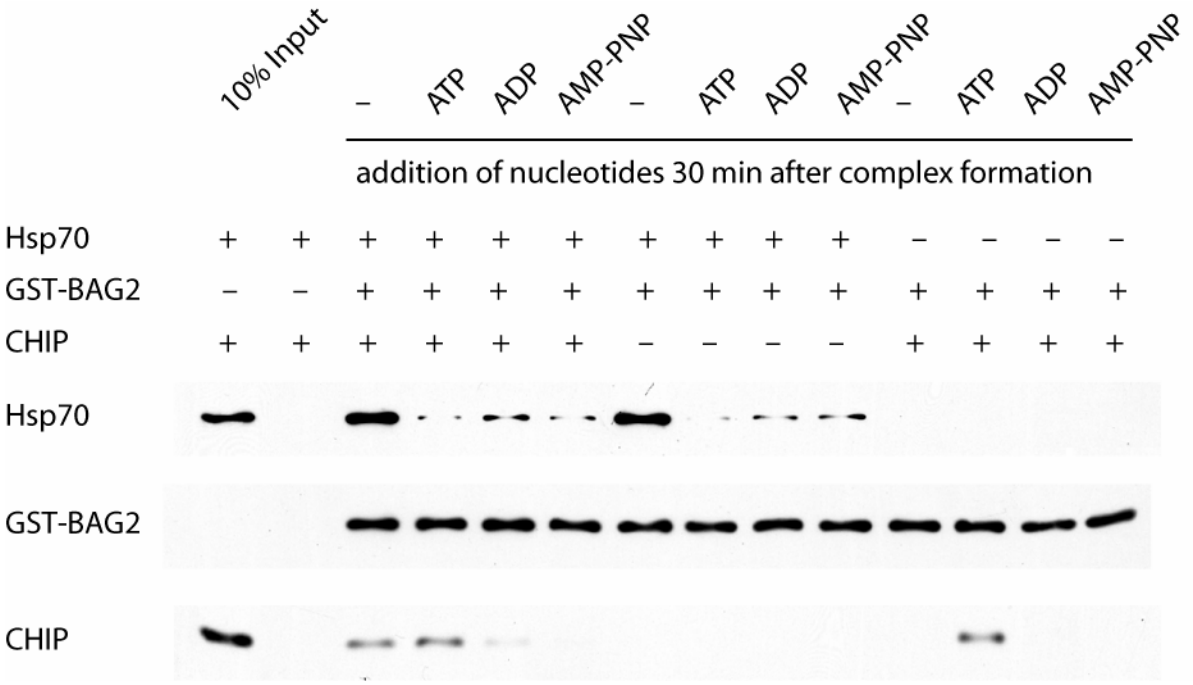
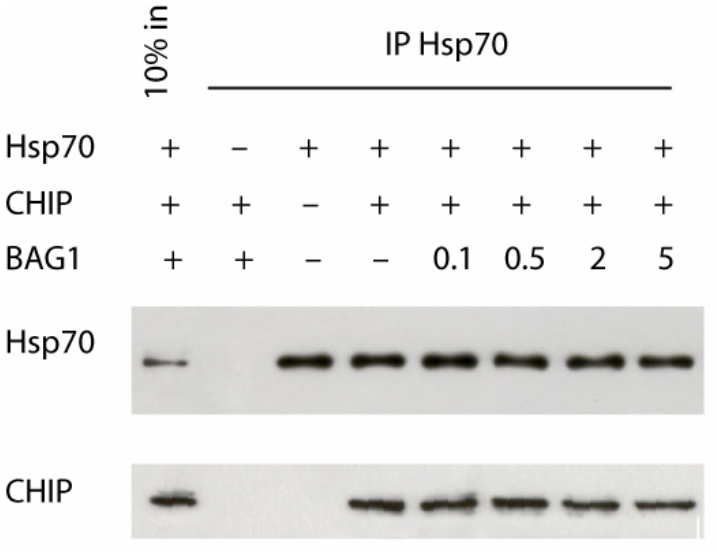


Figure 3.10 Nucleotide dependent remodeling of CHIP-Hsp70-BAG2 complexes. GST-BAG2, Hsp70 and CHIP were preassembled into complexes in different combinations for 30 min and then incubated with ATP, ADP, or the non-hydrolyzable ATP analog AMP-PNP. BAG2-containing complexes were then precipitated with glutathione-sepharose and Western blotting was performed to detect Hsp70 and CHIP in these complexes.

A



B

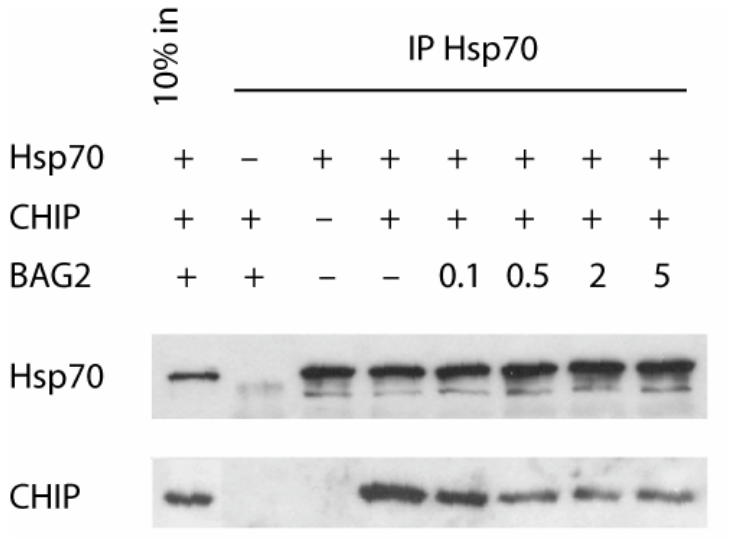


Figure 3.11 Dissociation of Hsp70 and CHIP by BAG2 in the presence of ATP. *A* and *B.* In vitro binding reactions containing 4 μM CHIP, 2 μM Hsp70, 4 μM Hdj2, 0.3 μg purified rabbit E1, 1 mg/ml ubiquitin, 8 μM UbcH4, and the indicated molar ratios of BAG1 (*A.*) or BAG2 (*B.*) were performed in 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 10 mM dithiothreitol. Hsp70 immunocomplexes were precipitated and blotted for the presence of CHIP and BAG2.

by coimmunoprecipitation. CHIP dimers were easily detected under these conditions, and BAG2 readily incorporated into complexes containing these dimers, but (even at saturating levels of BAG2 expression) CHIP dimerization was not affected (Figure 3.9).

Formation of Hsp70-containig protein complex is dependent on nucleotide binding, so we next examined the nucleotide dependence of the association of BAG2 and Hsp70 with CHIP. We observed that addition of adenine nucleotide induced release of Hsp70 from preformed CHIP-Hsp70-BAG2 complexes by GST-BAG2 pull-down assays; however, association of CHIP with BAG2 was retained in the presence of ATP but not ADP or the non-hydrolyzable ATP analog AMP-PNP, indicating that CHIP and BAG2 associated directly under this condition. This conclusion was supported by the experiment with incubation of BAG2 and CHIP only, which showed direct binding of BAG2 and CHIP in the presence of ATP (Figure 3.10). The release of Hsp70 from BAG2-CHIP complexes in the presence of ATP, as indicated in Figure 3.10, suggested that one mechanism to account for inhibition of CHIP by BAG2 is through dissociation of Hsp70 that is required for substrate presentation to CHIP. We therefore performed additional binding assays under the same conditions as our in vitro ubiquitylation reactions (Figure 3.11) in an ATP-containing system. Under these conditions, and in contrast to the effects observed in the absence of ATP (Figure 3.3-3.5), BAG2 disrupted the association between CHIP and Hsp70 (Figure 3.11A) in a concentration dependent manor that was analogous to its effect on CHIP ubiquitin ligase activity (Figure 3.7). In contrast, and in spite of the fact that it has equivalent effects on Hsp70 nucleotide exchange (Takayama and Reed, 2001), BAG1 had little effect on CHIP-Hsp70 interactions, even at relatively high molar ratios (Figure 3.11*B*), which is consistent with its inability to suppress CHIP E3 ligase activity toward NBD1 domain of CFTR

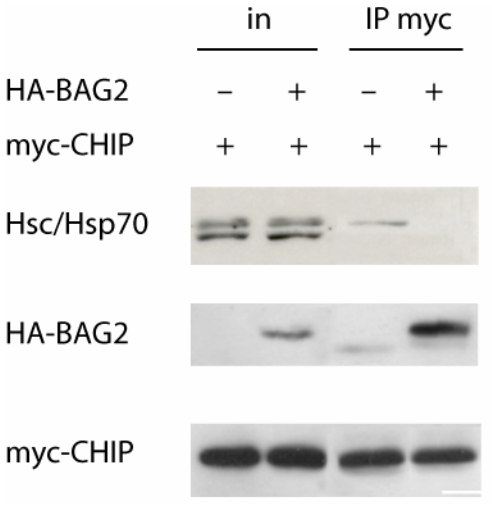


Figure 3.12 BAG2 induced dissociation of CHIP-Hsc/Hsp70 complexes in HeLa cells. Coimmunoprecipitation of Myc-tagged CHIP and endogenous Hsc/Hsp70 was performed in HeLa cells transfected with or without HA-tagged BAG2. CHIP immunoprecipitates were blotted for the presence of BAG2 and Hsc/Hsp70 with specific antibodies.

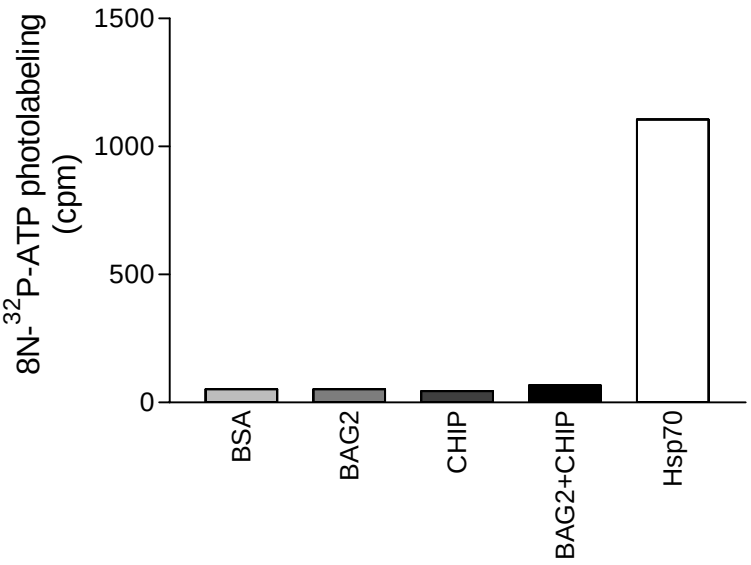


Figure 3.13 ATP binding of Hsp70, CHIP and BAG2. ATP binding was assayed by 8-azido-32P-ATP photolabeling of the indicated proteins. Only Hsp70, but not CHIP or BAG2 was able to bind to ATP.

(Figure 3.8). We also found that BAG2 disrupted the association of CHIP and endogenous Hsc/Hsp70 in vivo (Figure 3.12). Therefore, this ATP dependent remodeling of the CHIP-Hsp70 ubiquitin ligase complex into CHIP-BAG2 complex prevented Hsp70 to present substrates to CHIP, which results in the observed inhibition of the E3 ubiquitin ligase activity of CHIP (Figure 3.7).

The nucleotide dependence of this protein complex remodeling raised the question of which of these proteins bound to, and had their functions affected by, ATP. We tested this by 8-azido-ATP photolabeling, a sensitive index of ATP binding (Figure 3.13). As expected, 8-Azido-ATP photoreacted with Hsp70, but not with CHIP or BAG2. Similar results were observed with ^{32}P-ATP affinity chromatography (*not shown*). Taken together, these results suggests that remodeling of the Hsp70-BAG2-CHIP heterocomplex requires nucleotide binding to dissociate BAG2 from Hsp70 and (based on the effects of AMP-PNP) Hsp70-dependent nucleotide hydrolysis to facilitate the BAG2-CHIP interaction.

We have previously observed that stable interactions between CHIP and the ubiquitin conjugating enzyme UbcH5a are necessary for ubiquitin ligase activity (Jiang et al., 2001), we then examined whether direct interaction between CHIP and BAG2 had any effects on CHIP-UbcH5 interaction. In support of this hypothesis, we noted that incorporation of BAG2 into CHIP complexes in the presence of ATP destabilized this interaction (Figure 3.14), preventing E2–E3 coupling that is required for ubiquitin chain assembly. Given the proximity of BAG2 and CHIP within the chaperone/ubiquitin ligase holocomplex, this activity provides an additional mechanism for inhibition of chaperone substrate ubiquitylation. Taken together, our observations suggest that BAG2 regulates multiple aspects of the activity of CHIP-Hsp70 ubiquitin ligase complex, and thus exerts a checkpoint

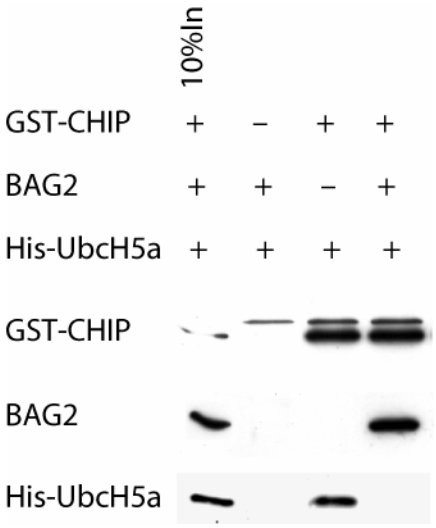


Figure 3.14 Disruption of CHIP E2-E3 coupling by CHIP. Interactions between UbcH5a (expressed as a His-tagged protein) with GST-CHIP were tested in the absence or presence of equimolar concentrations of BAG2. These binding reactions were performed in the presence of ATP (5 mM).

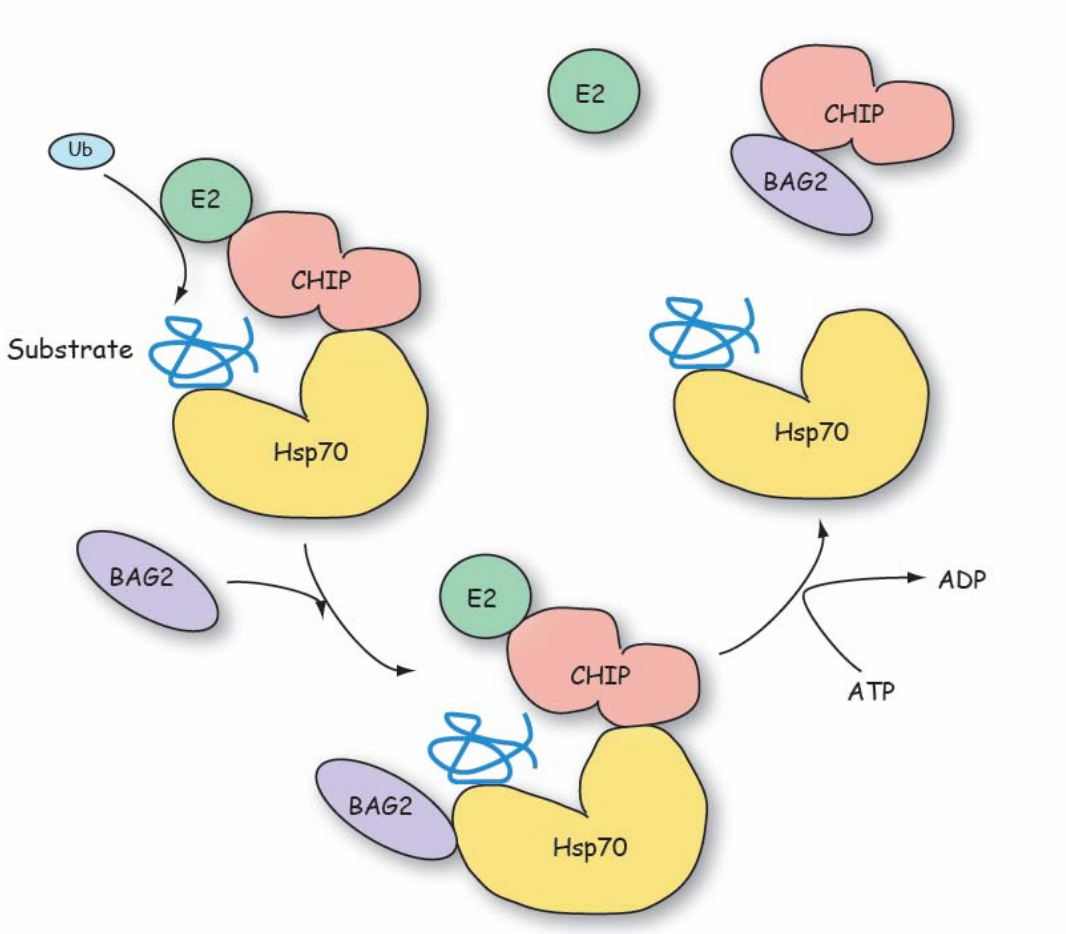


Figure 3.15 A model of the mechanism underlying the inhibitory effects of BAG2 on CHIP ubiquitin ligase activity. In the absence of BAG2, a holocomplex containing CHIP, Hsp70, an E2, and chaperone substrate assembles to facilitate substrate ubiquitylation. Through its BAG domain, BAG2 makes contact with the ATPase domain of Hsp70 and additional remodeling of the complex occurs in an ATP-dependent fashion that has 2 consequences. CHIP and BAG2 are dissociated from Hsp70, and binding of E2 to CHIP is uncoupled.

mechanism that may prevent dysregulated ubiquitylation of chaperone substrates.

3.5 Discussion

Protein quality control is essential for living cells to maintain their normal function and to cope with stress challenges. Molecular chaperones participate in both protein folding and degradation pathways of the cytoplasmic protein quality control system and their functions are regulated by a group of co-chaperones. One such co-chaperone, CHIP, inhibits the ATPase activity of Hsp70 in vitro, and activates HSF1 and increases the folding function of Hsp70 in vivo (Ballinger et al., 1999; Dai et al., 2003; Kampinga et al., 2003). Functioning in association with molecular chaperones Hsp70 and Hsp90, CHIP has also recently been shown to contains E3 ubiquitin ligase activity in facilitating chaperone substrate degradation (Connell et al., 2001; Meacham et al., 2001; Xu et al., 2002). Therefore, decision of whether to refold or to degrade chaperone substrates, termed as "protein triage", may be made by modulating functions of chaperone complexes. In order to understand regulation of Hsp70-CHIP complex, we immunopurified CHIP containing complexes in HeLa cell lines. We have found that BAG2 is a prominent component of CHIP-containing complexes, and it inhibits E3 ligase activity of the CHIP-Hsp70 complexes.

BAG2 is one of six proteins that contain carboxyl-terminal BAG domains in mammalian cells. BAG domains of BAG family proteins all interact with ATPase domain of Hsp70 (Takayama and Reed, 2001); they induce conformational change of this domain, which results in nucleotide exchange (Sondermann et al., 2001). Many of the BAG-domain proteins possess anti-apoptotic activities through different mechanisms. However, we did not detect any effects of BAG2 on apoptosis by using a variety of techniques (*data not shown*). What we do find is that BAG2, but not BAG1, induces dissociation of CHIP from Hsp70 and

release of E2 from CHIP, thus inhibits E3 ligase activity of CHIP-Hsp70 complex in the presence of ATP. This unique function of BAG2 may rely on the amino terminus of this protein, since the amino terminus of the BAG family proteins have diverse structure and function (Takayama et al., 1999). The amino-terminus of BAG2 is required to stabilize the ternary BAG2-Hsp70-CHIP complex, suggesting that these sequences may form protein-protein interactions within this complex—in addition to the BAG-ATPase domain interaction—to tune the molecular functions of this complex. Consistent with this model, ATP-dependent interaction of BAG2 with CHIP remodels the trimeric complexes so that Hsp70 is excluded (Figure 3.11). It is also plausible that the amino terminus of BAG2 sterically hinders interaction between UbcH5 and CHIP, although we can not exclude the possibility that BAG2 also has conformational effects on the complex that determine the rules of E2-E3 association (Figure 3.15).

Our work here provided a mechanism for the protein triage decision making by the inhibition of CHIP E3 ubiquitin ligase activity by BAG2. Our results indicate that BAG2 is likely to play a constitutive regulatory role in this decision, leaving regulation of this CHIP-BAG2 interaction an open question. CHIP-BAG2 interaction is dependent on nucleotide, so manipulation of nucleotide availability to the CHIP-Hsp70-BAG2 complexes might play an important regulatory role. Recently, BAG2 was identified as a phosphorylation target for p38 MAP kinase in a proteomic screen (Ueda et al., 2004); therefore, it is possible that functional regulation of BAG2 by phosphorylation might affect its association with CHIP-Hsp70 complexes. However, we did not find the effects of BAG2 on CHIP were altered by pharmacologic inhibitors or activators of p38 (*data not shown*). How the interaction of BAG2 with CHIP-Hsp70 is regulated remains to be elucidated.

Previous, the Hohfeld group reported that HspBP1 and BAG1 are able to affect the ubiquitin-proteasome degradation pathway mediated by CHIP. HspBP1 inhibited E3 ligase activity of CHIP, but with a mechanism different from disrupting E2-CHIP association (Alberti et al., 2004). BAG1 does not affect E3 ligase activity of CHIP, but stimulates CHIP-mediated degradation by the ability of BAG1 to associate with the proteasome using a ubiquitin-like domain at its amino terminus that is not present in BAG2 (Demand et al., 2001). We did not detect HspBP1 or BAG1 in our mass spectrometry experiment nor did the Hohfeld group that used a similar proteomic approach and identified BAG2, but not HspBP1 nor BAG1 in CHIP-containing protein complex (Arndt et al., 2005), suggesting that interaction between HspBP1 with CHIP-Hsp70 complex is highly regulated or is not a dominant interaction in the experimental system that we were using. Nevertheless, we propose two hypotheses about the antagonistic effects of BAG1 and BAG2 toward CHIP-dependent degradation. First, these two proteins may target different substrates of CHIP-Hsp70 complex, since BAG2 modulate the degradation of CFTR while BAG1 does not. Second, they may participate in serial interactions with CHIP-Hsp70 complex to regulate its function. Interaction of BAG2 with Hsp70 induces ATP binding, substrate loading, and inhibition of ubiquitin ligase activity. BAG1 binding would in turn relieve BAG2-dependent inhibition of ubiquitylation, and would concomitantly facilitate transfer of chaperone substrates ubiquitylated by CHIP to the proteasome for ATP dependent degradation; although the lower affinity of BAG1 compared with BAG2 would argue that this might occur only under specific circumstances. Further experiments are required to support these hypotheses. Taken together, our data suggest that BAG2 regulates the ubiquitin ligase

activity of Hsp70-CHIP complexes and therefore governs the balance between folding and degradation of chaperone substrates.

3.6 References

Alberti S, Bohse K, Arndt V, Schmitz A and Hohfeld J (2004) The Cochaperone HspBP1 Inhibits the CHIP Ubiquitin Ligase and Stimulates the Maturation of the Cystic Fibrosis Transmembrane Conductance Regulator. *Mol. Biol. Cell* **15**:4003-4010.

Arndt V, Daniel C, Nastainczyk W, Alberti S and Hohfeld J (2005) BAG-2 Acts as an Inhibitor of the Chaperone-associated Ubiquitin Ligase CHIP. *Mol. Biol. Cell*:E05-07-0660.

Ballinger CA, Connell P, Wu Y, Hu Z, Thompson LJ, Yin L-Y and Patterson C (1999) Identification of CHIP, a Novel Tetratricopeptide Repeat-Containing Protein That Interacts with Heat Shock Proteins and Negatively Regulates Chaperone Functions. *Mol. Cell. Biol.* **19**:4535-4545.

Borchers C, Peter J, Hall M, Kunkel T and Tomer K (2000) Identification of in-gel digested proteins by complementary peptide mass fingerprinting and tandem mass spectrometry data obtained on an electrospray ionization quadrupole time-of-flight mass spectrometer. *Anal Chem* **72**:1163-1168.

Connell P, Ballinger CA, Jiang J, Wu Y and Patterson C (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol.* **3**:93-96.

Dai Q, Zhang C, Wu Y, McDonough H, Whaley RA, Godfrey V, Li H-H, Madamanchi N, Xu W, Neckers L, Cyr D and Patterson C (2003) CHIP activates HSF1 and confers protection against apoptosis and cellular stress. *EMBO J.* **22**:5446-5458.

Demand J, Alberti S, Patterson C and Höhfeld J (2001) Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. *Curr Biol* **11**:1569-1577.

Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI and Takahashi R (2002) CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* **10**:55-67.

Jiang J, Ballinger CA, Wu Y, Dai Q, Cyr DM, Hohfeld J and Patterson C (2001) CHIP Is a U-box-dependent E3 Ubiquitin Ligase. IDENTIFICATION OF Hsc70 AS A TARGET FOR UBIQUITYLATION. *J. Biol. Chem.* **276**:42938-42944.

Kampinga HH, Kanon B, Salomons FA, Kabakov AE and Patterson C (2003) Overexpression of the Cochaperone CHIP Enhances Hsp70-Dependent Folding Activity in Mammalian Cells. *Mol. Cell. Biol.* **23**:4948-4958.

Meacham GC, Patterson C, Zhang W, Younger JM and Cyr DM (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol.* **3**:100-105.

Murata S, Minami Y, Minami M, Chiba T and Tanaka K (2001) CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.* **2**.

Nikolay R, Wiederkehr T, Rist W, Kramer G, Mayer MP and Bukau B (2004) Dimerization of the Human E3 Ligase CHIP via a Coiled-coil Domain Is Essential for Its Activity. *J. Biol. Chem.* **279**:2673-2678.

Sondermann H, Scheufler C, Schneider C, Hohfeld J, Hartl F and Moarefi I (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science* **291**:1553-1557.

Takayama S, Bimston D, Matsuzawa S, Freeman B, Aime-Sempe C, Xie Z, Morimoto R and Reed J (1997) BAG-1 modulates the chaperone activity of Hsp70/Hsc70. *Embo J* **16**:4887-4896.

Takayama S and Reed J (2001) Molecular chaperone targeting and regulation by BAG family proteins. *Nat Cell Biol.* **3**:E237-241.

Takayama S ST, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *cell* **80**:279-284.

Takayama S, Xie Z and Reed JC (1999) An Evolutionarily Conserved Family of Hsp70/Hsc70 Molecular Chaperone Regulators. *J. Biol. Chem.* **274**:781-786.

Ueda K, Kosako H, Fukui Y and Hattori S (2004) Proteomic Identification of Bcl2-associated Athanogene 2 as a Novel MAPK-activated Protein Kinase 2 Substrate. *J. Biol. Chem.* **279**:41815-41821.

Wickner S, Maurizi MR and Gottesman S (1999) Posttranslational Quality Control: Folding, Refolding, and Degrading Proteins. *Science* **286**:1888-1893.

Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C and Neckers L (2002) Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *PNAS* **99**:12847-12852.

Yaguchi H, Ohkura N, Takahashi M, Nagamura Y, Kitabayashi I and Tsukada T (2004) Menin Missense Mutants Associated with Multiple Endocrine Neoplasia Type 1 Are Rapidly Degraded via the Ubiquitin-Proteasome Pathway. *Mol. Cell. Biol.* **24**:6569-6580.

Younger JM, Ren H-Y, Chen L, Fan C-Y, Fields A, Patterson C and Cyr DM (2004) A foldable CFTR{Delta}F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. *J. Cell Biol.* **167**:1075-1085.

Chapter 4

Discussion and future directions

Molecular chaperones play a central role during stress response. They are induced upon stress challenges and bind to denatured proteins, helping them refolding and preventing them aggregating. Recently, it was suggested that they also assist the delivery of irreversibly damaged proteins for proteasomal degradation (Ellgaard and Helenius, 2001; Trombetta and Parodi, 2003). A protein triage model has been suggested for protein quality control. In that model, damaged proteins are recognized by molecular chaperones of the quality control system, and the competition between some E3 and E2 proteins of the ubiquitin-proteasome system and chaperones for binding to nonnative proteins determines the fate of refolding or degradation of those proteins (Wickner et al., 1999). However, recent work by the Patterson lab and other labs suggests that CHIP, a cochaperone that interacts with molecular chaperones Hsp70 and Hsp90 and increases the expression and refolding activity of chaperones in vivo (chapter two and (Kampinga et al., 2003)), also act as a chaperone-dependent ubiquitin E3 ligase that polyubiquitylates chaperone substrates for proteasomal degradation (Connell et al., 2001; Jiang et al., 2001; Meacham et al., 2001), and thus is a well suited candidate that helps to determine the fate of chaperone substrates. We propose that CHIP increases the overall folding capacity of the cells during stress response, but if a protein is severely damaged and is beyond repair, CHIP then remodels the chaperone system into part of the degradation machinery by polyubiquitylating the chaperone substrate and sending it to the proteasome. Therefore, CHIP plays a pivotal role in decision making of protein quality control.

4.1 Role of CHIP in the regulation of stress response

Induction of heat shock proteins is essential in stress response. Heat shock proteins buffer the deleterious effects of stress by binding to unfolded proteins and preventing them

accumulating. Functions of heat shock proteins are tightly controlled by a group of cochaperones. CHIP was identified as one such cochaperone that inhibits the ATPase activity of Hsp70 and induces the release of chaperone substrates. The consequence is inhibited Hsp70 refolding activity by CHIP in vitro (Ballinger et al., 1999). However, overexpression of CHIP enhances Hsp70-dependent folding activity in mammalian cells (Kampinga et al., 2003). Several explanations can reconcile the discrepancies between these two studies. In addition to the reasons presented in the introduction, in chapter II, we demonstrated that CHIP can induce the expression of Hsp70. Therefore, induction of Hsp70 by CHIP accounts for, at least partly, the increased overall folding activity of Hsp70 in vivo.

Heat shock response is not only associated with induction of Hsp70 and the increased refolding capacity of the cell, but also with the inhibition of transcription and translation of proteins in general and cell cycle arrest (Lindquist, 1986; Rowley et al., 1993; Luft et al., 2001). Although this may be beneficial for cells to cope with stress in the short term, unrestricted heat shock response is deleterious to the cells in the long run. The cells have evolved to use sophisticated mechanisms to keep the activity of HSF1 under tight control, including the regulation by Hsp70, Hsp90 and Hsp40, reversible phosphorylation and sumoylation, and sense heat and oxidative stress by HSF1 itself (see chapter one). In chapter II, we presented that CHIP interacts with Hsp70-HSF1 complex and remodels it to become a transcriptionally active complex that resists attenuation. In cells lacking endogenous CHIP, maximal induction of Hsp70 after heat shock is abolished. This work establishes an essential role of CHIP in the regulation of HSF1.

Several other proteins have been implicated in affecting the activity of HSF1. Daxx, a modulator of apoptosis and a repressor of basal transcription, is capable of interacting with

the trimeric form of human HSF1. This interaction leads to the activation of HSF 1 transcriptional activity and is required for maximal induction of Hsp70 after heat shock (Boellmann et al., 2004). Two lines of differences reside between the activation of HSF1 by CHIP and Daxx. First, CHIP interacts with Hsp70-HSF1 complexes before, during and after heat shock, although more CHIP protein is present in the Hsp70-HSF1 complexes during heat shock, while the nuclear protein Daxx only interacts with the trimeric form of HSF1 that has already bound to HSF. Second, overexpression of CHIP alone is sufficient to activate HSF1 and induce the expression of Hsp70, while overexpression of Daxx only minimally increases the transcription of Hsp70, but it induces transcriptional competence for pre-trimerization HSF1. Therefore, CHIP and Daxx represent two independent mechanisms that activate HSF1.

Two other proteins, Ral-binding protein 1 (RalBP1) and protein phosphatase 5 (PP5), have been reported as negative regulators of HSF1 activity. RalBP1 and HSF1 interact in vivo in a complex containing HSP90, α-tubulin and RalBP1·HSF1. Upon heat shock, the Ral signaling pathway is activated, resulting in the binding of RalBP1 by RalGTP. This is concurrent with the release of HSF1 from the RalBP1·HSF1·HSP90α-tubulin heterocomplexes, and translocation of HSF1 into the nucleus, where it then activates transcription (Hu and Mivechi, 2003). PP5, a TPR domain-containing component of Hsp90-steroid receptor complexes also interacts with HSF1-Hsp90 complexes. Overexpression of PP5 or activation of endogenous phosphatase activity results in diminished HSF1 DNA binding and transcriptional activities, and accelerated recovery. Depletion of PP5 by antibody or inhibition of its phosphatase activity in vivo significantly delays trimer disassembly after

heat shock (Conde et al., 2005). Inhibition of HSF1 by these two proteins represents the regulation of HSF1 by intracellular signaling pathways.

Regulation of HSF1 by proteins other than molecular chaperones represents a new mechanism for the regulation of HSF1 that is incorporated into the already complicated regulatory system. Although in each individual study, the factors reported (chaperones, phosphorylation sites, cysteine residues, CHIP, Daxx, etc) seem to be required for maximal induction or inhibition of HSF1 activity in their particular experimental system, the relative abundance of each regulator and activity of signaling pathways may determine the relative importance of each regulatory mechanism in different organs, tissues and cell types. The detailed mechanism for the regulation of HSF1 still needs to be elucidated.

We observed an antiapoptotic effect of CHIP. CHIP protects cultured fibroblasts from heat-induced apoptosis (Chapter II), and also protects cells of the gastrointestinal tract and spleen from apoptosis induced by thermal challenge of mice (Dai et al., 2003). Although the fact that CHIP coordinates folding, degradation and the global stress response, each of which probably contributes to its antiapoptotic effects, we were still interested in investigating if CHIP directly interferes with the apoptotic pathways.

ASK1 (Apoptosis signal-regulating kinase 1) is a MAPKKK (mitogen-activated protein kinase kinase kinase) that is regulated under conditions of cellular stress. ASK1 phosphorylates c-Jun N-terminal kinase (JNK) and elicits an apoptotic response. Because ASK1 is known to contain a TPR-acceptor site, our lab examined the role of CHIP in regulating ASK1 function. CHIP interacts with ASK1 in a TPR-dependent fashion and induces ubiquitylation and proteasome-dependent degradation of ASK1. Targeting of ASK1 by CHIP inhibits JNK activation in response to oxidative challenge and reduces ASK1-

dependent apoptosis. CHIP also triggers the translocation of ASK1 partner protein Daxx into the nucleus, where it is known to activate an antiapoptotic response. This work elucidated a mechanism for CHIP to interfere directly with and regulate the apoptotic pathway in the face of cellular and physiologic stress (Hwang et al., 2005).

CHIP is highly expressed in striated muscles (skeletal muscle and heart), making them the most appropriate targets to examine the endogenous function of CHIP in the setting of stress challenge. Our lab tested the response of CHIP(−/−) mice to in vivo ischemia and reperfusion injury induced by left anterior descending coronary artery ligation. We found that compared with their littermates, CHIP(−/−) mice have decreased survival and increased incidence of arrhythmias during reperfusion, which is accompanied by increased infarct size, impaired upregulation of Hsp70 and increased numbers of apoptotic cells in the myocardium and intramural vascular endothelium(Zhang et al., 2005). This work demonstrates that the chaperone system, including CHIP, plays a necessary role in protection against stress induced damages under pathophysiologically relevant conditions.

4.2 Role of CHIP as a chaperone dependent ubiquitin E3 ligase

Ever since the discovery that the U-box of CHIP has E3 ubiquitin ligase activity (Jiang et al., 2001; Murata et al., 2001), dozens of substrates have been assigned to CHIP, which can be broadly categorized into two groups: (1) signaling molecules, such as GR (Connell et al., 2001; Xin et al., 2005), unliganded ER (estrogen receptor) (Tateishi et al., 2004), Smad family proteins (Li et al., 2004), ErbB2 (Xu et al., 2002; Zhou et al., 2003), nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) (Bonvini et al., 2004), nNOS (Peng et al., 2004), and (2) mutant or aggregation-prone proteins such as mutant CFTR (Meacham et al., 2001; Younger et al., 2004), menin missense mutants (Yaguchi et al., 2004), wild type and mutant

p53 (Esser et al., 2005), and phosphorylated tau (Hatakeyama et al., 2004; Petrucelli et al., 2004; Shimura et al., 2004). The common feature of these proteins is that they are structurally labile and that they need to associate with molecular chaperones Hsp70 or Hsp90 to maintain their activation-competent state or to prevent them from aggregation. By polyubiquitylating and degrading these proteins, CHIP helps to modulate intracellular signaling and to regulate chaperone-dependent protein quality control.

As mentioned before, degradation of the first signaling molecule identified by CHIP is GR (Connell et al., 2001). Recently, estrogen-unbound (unliganded), especially misfolded ERα is found to be another CHIP substrate (Tateishi et al., 2004). These two proteins belong to the nuclear receptor superfamily. Upon binding to their ligands, they undergo a characteristic conformational change to gain DNA binding activity and activate transcription. Unliganded receptors are not stable and they need to associate with chaperones Hsp90 (GR) or Hsp90, Hsp70, and Hsp40 (ERα) to maintain their activation-competent state. Overexpression of CHIP induces degradation of GR and unliganded, especially misfolded ERα, while in cells lacking endogenous CHIP, degradation of unliganded ERα is blocked (Connell et al., 2001; Tateishi et al., 2004). These two studies suggest that CHIP regulates protein quality control of nuclear receptor family proteins, and at least for ERα, this regulation is relevant under physiological conditions with endogenous CHIP. CHIP has also been shown to decrease the steady-state protein levels of androgen receptor (He et al., 2004). It is therefore interesting to explore if CHIP has E3 ubiquitin ligase activity toward other nuclear receptor proteins.

Recently molecular chaperones have been implicated in facilitating malignant transformation by binding to overexpressed or structurally labile oncoproteins, thus buffering

genetic instability that is characteristic of many human cancers. Chaperone inhibitors, especially Hsp90 inhibitors geldanamycin and 17-AAG, are becoming a promising new family of anti-cancer drugs. By inhibiting Hsp90 function, Hsp90 inhibitors promote the destabilization and down-regulation of these oncoproteins that results in anti-cancer effect (Bagatell and Whitesell, 2004). Degradation of several Hsp90 bound oncoproteins, such as overexpressed ErbB2 (Xu et al., 2002; Zhou et al., 2003; Bagatell and Whitesell, 2004) and mutant p53 (Esser et al., 2005), is mediated by the E3 ligase activity of CHIP. In the presence of GA or 17-AAG, degradation of these oncoproteins is more prominent. Since Hsp90 has an essential role in signal transduction and protein folding, general inhibition of Hsp90 function leads to a profound adverse effect. Conjugation of Hsp90 inhibitor and CHIP might be a selective therapeutic approach for certain cancers. Also the expression level of CHIP might be an important determinant of the therapeutic success of such pharmacological interventions. Down-regulation of p53 by CHIP also provides a mechanism for CHIP-dependent anti-apoptotic effect during heat shock stress.

CFTR is a cytoplasmic membrane chloride channel, the mutation of which leads to cystic fibrosis. The biogenesis of CFTR is inefficient since 60-75% of the wild type and 99% of the mutant CFTR is degraded during their synthesis in the ER (Ward and Kopito, 1994). CHIP, Hsp70 and Hdj2 forms a ubiquitin ligase complex that polyubiquitylates and degrades immature CFTR during its synthesis in the ER. Inactivation of this E3 ligase complex leads to accumulation of the major mutant CFTRΔF508 in a nonaggregated state, which upon lowering of temperature, can fold and reach the cell surface. Therefore, although ER associated degradation of the biogenesis intermediate of the CFTR mutant prevents it from forming aggregates, it also prevents the folding-competent intermediate to mature to the cell

membrane that leads to the manifestation of the disease (Younger et al., 2004). Quality control of immature CFTR mediated by this CHIP-dependent E3 ligase complex results in a deleterious effect, and inhibition of this ubiquitin ligase provides a new therapeutic approach to treat CF.

The canonical ubiquitin-proteasome degradation system is characterized by polyubiquitin chain formation via lysine 48 linkage onto substrates that leads to their proteasomal degradation. Exceptions occur in this system, including for CHIP substrates. Linkages of polyubiquitin chain other than lysine 48 occur on CHIP substrates. Hsc70, the original CHIP substrate identified, is polyubiquitylated via either lysine 29 or lysine 63 linkages, and polyubiquitylation of Hsc70 does not result in degradation, but is of an unknown function (Jiang et al., 2001). The cochaperone BAG1, which enhances CHIP substrate degradation by presenting CHIP substrates to the proteasome via its ubiquitin-like domain (Demand et al., 2001), is ubiquitylated by CHIP through lysine 11 (Alberti et al., 2002). This ubiquitylation does not induce degradation of BAG1, but stimulates the association of BAG1 with the proteasome (Alberti et al., 2002). Thus, BAG1 ubiquitylation, in conjugation with Hsc70 ubiquitylation, may act as a sorting signal for delivering polyubiquitylated CHIP substrates to the proteasome.

Polyubiquitylation of substrates by CHIP does not always results in degradation. Epsin, an endocytic adaptor protein that is involved in the regulation of clathrin-dependent endocytosis, is polyubiquitylated by CHIP. However, polyubiquitylation of Epsin by CHIP does not require molecular chaperones Hsp70 nor Hsp40, and polyubiquitylated Epsin does not lead to proteasomal degradation (Timsit et al., 2005). Like Hsp70 ubiquitylation, the function of Epsin ubiquitylation by CHIP is unknown.

The expression of CHIP is not ubiquitous, but restricted to a few organs. In addition to striated muscles, the brain is the second organ that CHIP is highly expressed (Ballinger et al., 1999). Given the role that CHIP plays in protein quality control, it is not surprising that CHIP is implicated in a variety of neurodegenerative diseases, such as Alzheimer' disease (AD) (Petrucelli et al., 2004; Shimura et al., 2004; Sahara et al., 2005), amyotrophic lateral sclerosis (ALS) (Urushitani et al., 2004), and familial Parkinson's disease (PD) (Imai et al., 2002).

There is no consensus of the role CHIP plays in tau-associated neurodegeneration diseases. Aggregation of hyperphosphorylated, microtubule-associated protein tau is characteristic of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. However, it is not clear whether hyperphosphrylated soluble tau, or the polyubiquitylated tau aggregates, is toxic to neurons. Overexpression of CHIP leads to polyubiquitylation of hyperphosporylated tau, which results in increased degradation (Shimura et al., 2004) or aggregation (Petrucelli et al., 2004) of tau proteins, and increased cell survival (Shimura et al., 2004). An in vivo study shows that CHIP proteins levels are inversely proportional to tau accumulates in AD brain samples. In aged mice lacking CHIP, there is an increased level of insoluble tau (Sahara et al., 2005). Taken together, these studies suggest that by polyubiquitylating hyperphosphorylated tau proteins, CHIP exerts a neuronal protective role in taupathies. Examination of CHIP knock out mice may lead to the understanding of the pathogenesis of taupathies.

CHIP plays an additional role in the ubiquitin-proteasome pathway. UFD2 is the yeast U-box protein classified as an E4 ubiquitin chain elongation factor. UFD2 cannot cooperate directly with E2 enzymes to ubiquitylate substrates, but functions in conjunction with an E3

ligase to elongate the polyubiquitin chain on substrates (Koegl et al., 1999). The U-box of CHIP contains such E4 activity during the degradation of Pael receptor (Pael-R) by the RING-finger containing E3 ligase Parkin, whose mutation is responsible for familial Parkinson's disease. CHIP forms a complex with Hsp70, Parkin and Pael-R and promotes the dissociation of Hsp70 from Parkin and Pael-R, thus facilitating and enhancing Parkin-mediated Pael-R ubiquitylation (Imai et al., 2002). CHIP and Hsc70 also forms a preubiquitylation complex (PUC) with E47 and Skp2 and facilitates the ubiquitylation and degradation of E47 by Skp2 during lymphocyte development (Huang et al., 2004). Both the E4 activity and the formation of PUC facilitate substrate ubiquitylation, thus CHIP can act as a cofactor for the ubiquitin-proteasome pathway to facilitate protein quality control in addition to being an E3 ligase.

4.3 Other functions of CHIP in protein quality control

Functions of CHIP other than the regulation of stress response and substrate ubiquitylation have emerged. CHIP can interfere with intracellular trafficking. CHIP interacts with Hsp90/eNOS complex and decreases the soluble eNOS levels. However, CHIP does not ubiquitylate eNOS and induce its degradation, but interferes with the trafficking of eNOS from the Golgi apparatus to the plasma membrane. Therefore eNOS is partitioned into an inactive intracellular compartment rather than mature through the Golgi apparatus and arrive onto the plasma membrane (Jiang et al., 2003). This represents a novel mechanism for CHIP to down-regulate chaperone substrates, which is distinct from the utilization of the ubiquitin-proteasome system. What the physiological significance of this regulation is and whether other chaperone substrates are regulated by a similar mechanism remain to be elucidated.

4.4 Regulation of CHIP function

Given the importance of CHIP in protein quality control, it is necessary to tightly control the activity of CHIP to prevent unnecessary activation of the stress response or global diversion of chaperone substrates to the proteasomal degradation pathway. The regulation of CHIP activity is not completely understood. The steady-state protein levels of CHIP are largely unchanged during stress challenges in cultured cells and in mouse tissue ((Tateishi et al., 2004) and chapter two), except the observation that CHIP protein levels are upregulated during ER stress induced by tunicamycin in a neuroblastoma cell line (Imai et al., 2002). Therefore several labs set out to examine the functional regulation of CHIP.

CHIP is posttranslationally phosphorylated on serine and threonine residues (Hwang and Patterson, unpublished observation). However, how these phosphorylations affect CHIP function and the kinases that responsible for these phosphorylations are unclear.

CHIP forms homodimers at physiological concentrations. The central charged region that is predicted to form coiled-coil domain is necessary and sufficient for dimer formation. A CHIP mutant that lacks this segment also loses its E3 ubiquitin ligase activity. This is the first evidence that the function of CHIP can be regulated by manipulation of its dimerization states (Nikolay et al., 2004). However, although the authors claim that the large deletion mutation of hCHIPdelta-(128-229) does not affect the structure of the remaining TPR or U-box domain, and thus the loss of E3 ligase activity is due to the inability to dimerize, it is plausible that such a large deletion to a relatively small protein (about one third of the full-length protein) might have a profound effect on the functional integrity of this protein. One possible effect might be the bringing of the TPR domain and the U-box close together, therefore generating steric hindrance that prevents the interaction of the U-box with E2 enzyme. Point mutations in the proposed dimerization domain need to be examined to

understand the residues that are important for dimerization and its effects on CHIP E3 ligase activity.

During my study of CHIP function, I set out to detect protein factors that associate with CHIP and the functional consequences of this interaction. I found that BAG2 is a major component of CHIP containing complex. Hsp70 serves as a bridge that forms a BAG2-Hsp70-CHIP complex. In the presence of ATP, BAG2 associates directly with CHIP, which disrupts CHIP-Hsp70 and CHIP-E2 interaction and leads to inhibition of CHIP E3 ligase activity. Thus BAG2 acts an inhibitor of CHIP E3 ligase activity (chapter 3). It has been previously reported that HspBP1 inhibits E3 activity of CHIP toward the Hsc/Hsp70 bound substrates of Raf1, denatured luciferase, and CFTR, and promotes CFTR maturation (Alberti et al., 2004). Two lines of differences exist between BAG2 and HspBP1 in their regulation of CHIP. BAG2 is much more abundant in the CHIP containing complex than HspBP1. BAG2 is a major band that is readily detectable from the CHIP IP complexes resolved by SDS-PAGE and silver staining, while HspBP1 can only be detected by Western blotting of the CHIP containing complexes (chapter 3 and (Alberti et al., 2004)). This suggests that BAG2 is a major regulator of CHIP function at physiological conditions, while HspBP1 might become a major regulator under certain circumstances that its interaction with CHIP is upregulated, or in certain tissues that CHIP-HspBP1 complexes are abundant. Second, they have different mechanism of inhibition. BAG2 disrupts the CHIP-Hsp70-E2 ubiquitylation machinery (chapter 3 and (Alberti et al., 2004)), while HspBP1 does not, but may induce conformational change of the E3 complex (Alberti et al., 2004). It is interesting to note that when we submitted our manuscript of the CHIP-BAG2 interaction story for review, we were informed that the Hehfeld group submitted a similar manuscript to the same journal. Fortunately, our

paper was published before theirs. I was surprised to find the strikingly similarity between the two papers when their paper was published (Arndt et al., 2005), which indicates the reproducibility of our data. Degradation of chaperone substrates by CHIP constitutively is a heavy burden for the cells because vast amount of energy is wasted when used for synthesis of these proteins. Here the work of the Hehfeld group and ours suggests that additional protein co-factors help to regulate E3 ligase activity of CHIP and determine the fate of its substrates.

Previously the Hehfeld group reported that BAG1 promotes CHIP substrate degradation. BAG1 does not affect E3 ligase activity of CHIP, but facilitates polyubiquitylated CHIP substrates to the proteasome, which is mediated by the association of the ubiquitin-like domain of BAG1 to the proteasome (Demand et al., 2001).

To date, BAG2, HspBP1 and BAG1 are the only proteins that have been identified to modulate the function of CHIP in the ubiquitin-proteasome degradation pathway. The common feature of these proteins is that they are all Hsp70 cochaperones that regulate Hsp70 nucleotide states. It is conceivable that since they have affinity for Hsp70, they gain access to the CHIP-Hsp70 ubiquitin ligase complex and modulate its activity. It is interesting to explore if other Hsp70 cochaperones have similar activity toward the CHIP-Hsp70 ubiquitin ligase dependent protein degradation pathway.

4.5 Future directions

We now know that CHIP is a link between the chaperone and the proteasome system, and thus plays an important role in protein quality control. As our knowledge about the function of CHIP increases exponentially, more questions are generated during the course of investigation:

1. Regulation of stress response. We have shown that CHIP forms a complex with Hsp70-HSF1 that is transcriptionally competent. Since protein levels of CHIP are largely unchanged during stress, what signals CHIP to associate with Hsp70-HSF1 and activate transcription? Does CHIP sense heat stress itself or other signals such as dimerization or phosphorylation of CHIP serve as a signal? What suppresses the activity of this complex when stress signals subside?

2. CHIP acts as a chaperone-dependent E3 ligase. CHIP polyubiquitylates dozens of, but not all chaperone substrate. What determines the substrate specificity of CHIP? What determines the lysine residues of ubiquitin that are used to form polyubiquitin chain on substrates by CHIP? What determines the fates of polyubiquitylated CHIP substrates, to the degradation or degradation-independent pathway? What is the function of CHIP in the pathogenesis of polyQ diseases and taupathies?

3. Regulation of CHIP. CHIP regulates both arms of protein triage by enhancing the refolding capacity of the cell and by diverting chaperone substrates to the degradation machinery. The central question to this system is how CHIP determines to refold some chaperone substrates while degrading others. Is it affected by nucleotide binding states of chaperones, association with other cochaperones, or regulation of CHIP function? How does phosphorylation or other posttranslational modifications regulate CHIP function? What are the enzymes responsible for and what regulates these modifications? Is dimerization of CHIP important for its function? What functions of CHIP are affected by modulating its dimerization states? What determines the dimerization states of CHIP? Since CHIP-Hsp70-BAG2 form a stable complex that is not affected by stresses such as heat shock, what is the

physiological importance of this regulation? How is inhibitory activity of BAG2 on CHIP regulated during normal and stress conditions? Are there other regulators of CHIP?

4.5 References

Alberti S, Bohse K, Arndt V, Schmitz A and Hohfeld J (2004) The Cochaperone HspBP1 Inhibits the CHIP Ubiquitin Ligase and Stimulates the Maturation of the Cystic Fibrosis Transmembrane Conductance Regulator. *Mol. Biol. Cell* **15**:4003-4010.

Alberti S, Demand J, Esser C, Emmerich N, Schild H and Hohfeld J (2002) Ubiquitylation of BAG-1 Suggests a Novel Regulatory Mechanism during the Sorting of Chaperone Substrates to the Proteasome. *J. Biol. Chem.* **277**:45920-45927.

Arndt V, Daniel C, Nastainczyk W, Alberti S and Hohfeld J (2005) BAG-2 Acts as an Inhibitor of the Chaperone-associated Ubiquitin Ligase CHIP. *Mol. Biol. Cell*:E05-07-0660.

Bagatell R and Whitesell L (2004) Altered Hsp90 function in cancer: A unique therapeutic opportunity. *Mol Cancer Ther* **3**:1021-1030.

Ballinger CA, Connell P, Wu Y, Hu Z, Thompson LJ, Yin L-Y and Patterson C (1999) Identification of CHIP, a Novel Tetratricopeptide Repeat-Containing Protein That Interacts with Heat Shock Proteins and Negatively Regulates Chaperone Functions. *Mol. Cell. Biol.* **19**:4535-4545.

Boellmann F, Guettouche T, Guo Y, Fenna M, Mnayer L and Voellmy R (2004) DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. *PNAS* **101**:4100-4105.

Bonvini P, Rosa HD, Vignes N and Rosolen A (2004) Ubiquitination and Proteasomal Degradation of Nucleophosmin-Anaplastic Lymphoma Kinase Induced by 17-Allylamino-Demethoxygeldanamycin: Role of the Co-Chaperone Carboxyl Heat Shock Protein 70-Interacting Protein. *Cancer Res* **64**:3256-3264.

Conde R, Xavier J, McLoughlin C, Chinkers M and Ovsenek N (2005) Protein Phosphatase 5 Is a Negative Modulator of Heat Shock Factor 1. *J. Biol. Chem.* **280**:28989-28996.

Connell P, Ballinger CA, Jiang J, Wu Y and Patterson C (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol.* **3**:93-96.

Dai Q, Zhang C, Wu Y, McDonough H, Whaley RA, Godfrey V, Li H-H, Madamanchi N, Xu W, Neckers L, Cyr D and Patterson C (2003) CHIP activates HSF1 and confers protection against apoptosis and cellular stress. *EMBO J.* **22**:5446-5458.

Demand J, Alberti S, Patterson C and Höhfeld J (2001) Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. *Curr Biol* **11**:1569-1577.

Ellgaard L and Helenius A (2001) ER quality control: towards an understanding at the molecular level. *Curr Opin Cell Biol.* **13**:431-437.

Esser C, Scheffner M and Hohfeld J (2005) The Chaperone-associated Ubiquitin Ligase CHIP Is Able to Target p53 for Proteasomal Degradation. *J. Biol. Chem.* **280**:27443-27448.

Hatakeyama S, Matsumoto M, Kamura T, Murayama M, Chui D-H, Planel E, Takahashi R, Nakayama KI and Takashima A (2004) U-box protein carboxyl terminus of Hsc70-interacting protein (CHIP) mediates poly-ubiquitylation preferentially on four-repeat Tau and is involved in neurodegeneration of tauopathy. *Journal of Neurochemistry* **91**:299-307.

He B, Bai S, Hnat AT, Kalman RI, Minges JT, Patterson C and Wilson EM (2004) An Androgen Receptor NH2-terminal Conserved Motif Interacts with the COOH Terminus of the Hsp70-interacting Protein (CHIP). *J. Biol. Chem.* **279**:30643-30653.

Hu Y and Mivechi NF (2003) HSF-1 Interacts with Ral-binding Protein 1 in a Stress-responsive, Multiprotein Complex with HSP90 in Vivo. *J. Biol. Chem.* **278**:17299-17306.

Huang Z, Nie L, Xu M and Sun X-H (2004) Notch-Induced E2A Degradation Requires CHIP and Hsc70 as Novel Facilitators of Ubiquitination. *Mol. Cell. Biol.* **24**:8951-8962.

Hwang J, Zhang C and Patterson C (2005) C-terminus of heat shock protein 70--interacting protein facilitates degradation of apoptosis signal-regulating kinase 1 and inhibits apoptosis signal-regulating kinase 1--dependent apoptosis. *cell Stress Chaperones* **10**:147-156.

Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI and Takahashi R (2002) CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* **10**:55-67.

Jiang J, Ballinger CA, Wu Y, Dai Q, Cyr DM, Hohfeld J and Patterson C (2001) CHIP Is a U-box-dependent E3 Ubiquitin Ligase. IDENTIFICATION OF Hsc70 AS A TARGET FOR UBIQUITYLATION. *J. Biol. Chem.* **276**:42938-42944.

Jiang J, Cyr D, Babbitt RW, Sessa WC and Patterson C (2003) Chaperone-dependent Regulation of Endothelial Nitric-oxide Synthase Intracellular Trafficking by the Co-chaperone/Ubiquitin Ligase CHIP. *J. Biol. Chem.* **278**:49332-49341.

Kampinga HH, Kanon B, Salomons FA, Kabakov AE and Patterson C (2003) Overexpression of the Cochaperone CHIP Enhances Hsp70-Dependent Folding Activity in Mammalian Cells. *Mol. Cell. Biol.* **23**:4948-4958.

Koegl M, Hoppe T, Schlenker S, Ulrich HD, Mayer TU and Jentsch S (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. C*ell* **96**:635-644.

Li L, Xin H, Xu X, Huang M, Zhang X, Chen Y, Zhang S, Fu X-Y and Chang Z (2004) CHIP Mediates Degradation of Smad Proteins and Potentially Regulates Smad-Induced Transcription. *Mol. Cell. Biol.* **24**:856-864.

Lindquist S (1986) The heat-shock response. *Annu Rev Biochem* **55**:1151-1191.

Luft J, Benjamin I, Mestril R and Dix D (2001) Heat shock factor 1-mediated thermotolerance prevents cell death and results in G2/M cell cycle arrest. *Cell Stress Chaperones* **6**:326-336.

Meacham GC, Patterson C, Zhang W, Younger JM and Cyr DM (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol.* **3**:100-105.

Murata S, Minami Y, Minami M, Chiba T and Tanaka K (2001) CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.* **2**:1133-1138.

Nikolay R, Wiederkehr T, Rist W, Kramer G, Mayer MP and Bukau B (2004) Dimerization of the Human E3 Ligase CHIP via a Coiled-coil Domain Is Essential for Its Activity. *J. Biol. Chem.* **279**:2673-2678.

Peng H-M, Morishima Y, Jenkins GJ, Dunbar AY, Lau M, Patterson C, Pratt WB and Osawa Y (2004) Ubiquitylation of Neuronal Nitric-oxide Synthase by CHIP, a Chaperone-dependent E3 Ligase. *J. Biol. Chem.* **279**:52970-52977.

Petrucelli L, Dickson D, Kehoe K, Taylor J, Snyder H, Grover A, De Lucia M, McGowan E, Lewis J, Prihar G, Kim J, Dillmann WH, Browne SE, Hall A, Voellmy R, Tsuboi Y, Dawson TM, Wolozin B, Hardy J and Hutton M (2004) CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. *Hum. Mol. Genet.* **13**:703-714.

Rowley A, Johnston G, Butler B, Werner-Washburne M and Singer R (1993) Heat shock-mediated cell cycle blockage and G1 cyclin expression in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* **13**:1034-1041.

Sahara N, Murayama M, Mizoroki T, Urushitani M, Imai Y, Takahashi R, Murata S, Tanaka K and Takashima A (2005) In vivo evidence of CHIP up-regulation attenuating tau aggregation. *J Neurochem.* **94**:1254-1263.

Shimura H, Schwartz D, Gygi SP and Kosik KS (2004) CHIP-Hsc70 Complex Ubiquitinates Phosphorylated Tau and Enhances Cell Survival. *J. Biol. Chem.* **279**:4869-4876.

Tateishi Y, Kawabe Y, Chiba T, Murata S, Ichikawa K, Murayama A, Tanaka K, Baba T, Kato S and Yanagisawa J (2004) Ligand-dependent switching of ubiquitin-proteasome pathways for estrogen receptor. *Embo J* **23**:4813-4823.

Timsit Y, Miller S, Mohney R and O'Bryan J (2005) The U-box ligase carboxyl-terminus of Hsc 70-interacting protein ubiquitylates Epsin. *Biochemical and Biophysical Research Communications* **328**:550-559.

Trombetta ES and Parodi AJ (2003) QUALITY CONTROL AND PROTEIN FOLDING IN THE SECRETORY PATHWAY. *Annual Review of Cell and Developmental Biology* **19**:649-676.

Urushitani M, Kurisu J, Tateno M, Hatakeyama S, Nakayama K-I, Kato S and Takahashi R (2004) CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70. *Journal of Neurochemistry* **90**:231-244.

Ward CL and Kopito RR (1994) Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J. Biol. Chem.* **269**:25710-25718.

Wickner S, Maurizi MR and Gottesman S (1999) Posttranslational Quality Control: Folding, Refolding, and Degrading Proteins. *Science* **286**:1888-1893.

Xin H, Xu X, Li L, Ning H, Rong Y, Shang Y, Wang Y, Fu X-Y and Chang Z (2005) CHIP Controls the Sensitivity of Transforming Growth Factor-{beta} Signaling by Modulating the Basal Level of Smad3 through Ubiquitin-mediated Degradation. *J. Biol. Chem.* **280**:20842-20850.

Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C and Neckers L (2002) Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *PNAS* **99**:12847-12852.

Yaguchi H, Ohkura N, Takahashi M, Nagamura Y, Kitabayashi I and Tsukada T (2004) Menin Missense Mutants Associated with Multiple Endocrine Neoplasia Type 1 Are Rapidly Degraded via the Ubiquitin-Proteasome Pathway. *Mol. Cell. Biol.* **24**:6569-6580.

Younger JM, Ren H-Y, Chen L, Fan C-Y, Fields A, Patterson C and Cyr DM (2004) A foldable CFTR{Delta}F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. *J. Cell Biol.* **167**:1075-1085.

Zhang C, Xu Z, He X-R, Michael LH and Patterson C (2005) CHIP, a cochaperone/ubiquitin ligase that regulates protein quality control, is required for maximal cardioprotection after myocardial infarction in mice. *Am J Physiol Heart Circ Physiol* **288**:H2836-2842.

Zhou P, Fernandes N, Dodge IL, Reddi AL, Rao N, Safran H, DiPetrillo TA, Wazer DE, Band V and Band H (2003) ErbB2 Degradation Mediated by the Co-chaperone Protein CHIP. *J. Biol. Chem.* **278**:13829-13837.